A transgenic mouse model for evaluation of the function of the NF-kappaB-repressing factor NRF

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Publikationen

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Tagungsbeiträge

Froese N., Kröger A. and Hauser H. (2004). Method for rapid *In vitro* excision of floxed genes by retroviral Cre transduction. (Poster) 5th VW Stiftung Meeting on Conditional Mutagenesis and Mouse Models, Dresden 2004.

Abstract

The NF-κB repressing factor (NRF) was proposed to act as inhibitor in some NF-κB regulated promoters. NRF was shown to have inhibitory effects on IFN-β, human iNOS and IL-8 promoters. These studies were carried out in established cell lines. To investigate the physiological function of NRF *in vivo,* technology of homologous recombination in mouse ES cells followed by injection into a blastocysts was applied and mice lacking NRF protein were created. NRF-KO mice are viable, appear phenotypically normal and exhibit normal life spans.

To elucidate the effects of NRF protein deletion on the molecular level different cell types lacking NRF expression were established and analysed. In none of tested cells the expected expression of the cytokine genes (IFN-β and iNOS) was detected in the non-induced state.

To examine a possible compensation of the lack of NRF during embryonal development a method for gene deletion in primary cells was established. Efficient infection was done with a retrovirus that leads to constitutive expression of GFP-Cre fusion protein. Homogenous population of NRF-deleted cells was obtained by cell sorting shortly after infection. This sudden deletion of the NRF gene did not provoke an induction of the IFN-β promoter. The presence of one or more factors that function on the inhibition of constitutive activity of this cytokine to rapidly compensate the lack of NRF is proposed.

However the expression of numerous genes affected by changes in NRF expression was determined by gene expression profiling. The numerous genes which expression was affected belong to different function groups and are involved in various cellular processes like apoptosis and immune response.

Contents

1. Introduction

1.1 Nuclear factor kappa B (NF-κB)

For their survival, all organisms have developed endogenous mechanisms to respond to different kinds of stress. These stress responses are closely linked to various signal transduction pathways so that the expression of a wide spectrum of proteins related to survival of the organism are effectively controlled. NF-κB has been identified as one of the critical transcription factors required for expression of genes regulating inflammation, proliferation and cell death. Transcription of pro-inflammatory molecules such as the cytokines TNF-α, IFN-α/β, IL-1, IL-2, IL-6, IL-12, LTα/β and GM-CSF, the chemokines IL-8, RANTES, MIP-1α, MCP1 and endotoxin, the adhesion molecules ICAM-1, VCAM, E-selectin, the acute phase proteins (SAA) and pro-inflammatory enzymes like iNOS (inducible nitric oxide synthase), haem oxygenase, cyclooxygenase-2 (COX-2) and xanthine dehydrogenase are regulated by NF-κB. First described in 1986 as a nuclear factor necessary for immunoglobulin κ light chain transcription in B cells (Sen and Baltimore, 1986), NF-κB is known to exist in virtually all cell types (Ghosh and Karin, 2002).

NF-κB proteins

NF-κB are heterodimers that monomers belong to a family of related proteins. There are five NF-κB/REL genes, NFKB1, NFKB2, REL-A, c-REL and RELB, that give rise to the proteins: REL-A (p65), NF-κB1 (p50, p105), NF-κB2 (p52, p100), c-REL and REL-B. These proteins have a structurally conserved amino-terminal 300-amino-acid region, which contains the domain responsible for dimerization, nuclear-localization and DNA-binding. The c-REL, REL-B and REL-A proteins also have a carboxy-terminal but non-homologous transactivation domain, which strongly activates transcription from NF-κB-binding sites in target genes. The other REL proteins, such as p50, lack the transactivation domain, but they still bind to NF-κB consensus sites in DNA and, therefore, function as transcriptional repressors (May and Ghosh, 1997). The p50 and p52 proteins are generated by proteolytic processing of precursor p105 and p100 proteins, respectively. Each NF-κB/REL-family member participates in the formation of different homo- or heterodimers with another family member. The predominant activated form of NF-κB is a heterodimer composed of the p65 subunit associated with either

a p50 or a p52 subunit. Whereas p50 and p65 are expressed widely in various cell types, the expression of REL-B is restricted to specific regions of the thymus and lymph nodes. The expression of c-REL is confined to haematopoietic cells and lymphocytes.

Genes that encode all five members of the NF-κB family have been deleted by homologous recombination in mice (Gerondakis et al, 1999). These gene-knockout animal models indicate the distinct roles of the NF-κB proteins in the regulation of innate and adaptive immune responses, lymphocyte function and cell survival (Li and Verma, 2002; table 1.1). Mice that lack more than one member of the NF-κB family ($p50^{-/-}RelB^{-/-}$ and $p50^{-/-}p52^{-/-}$) have more severe phenotypes, which indicates that there is functional redundancy between the NF-κB family members.

Mutated gene product	Phenotype in knockout mice
p65 (Rel-A)	Die at E15.5–E16.5; TNF-dependent liver apoptosis; defect in lymphocyte activation
NF-κB1 (both p105 and p50)	Survival to adulthood; defect in lymphocyte activation
NF-κB2 (both p100 and p52)	Survival to adulthood; no mature B cells and defect in lymphocyte activation; disruption of splenic and lymph-node architecture
Rel-B	Die postnatally from multi-organ inflammation; required for dendritic cell development
c-Rel	No developmental defects; defects in lymphocyte and macrophage functions
p65 and NF-κB1	Die at E13.5–E14.5
NF-κB1 and NF-κB2	Die postnatally; lack mature B cells and osteoblasts
NF-κB1 and Rel-B	Die postnatally owing to immune deficiency

Table 1.1. *Phenotype of knockout mice for NF-κB signalling components (Li and Verma, 2002).*

IκB proteins

NF-κB proteins exist in the cytoplasm in an inactive form as a result of their association with the IκB proteins of which the most common are IκBα, IκBβ and IκBε (Ghosh et al, 1998). In the cytoplasm, NF-κB is effectively sequestered by IκB in an inactive state via the ability of IκB to mask the NLS of NF-κB. NF-κB activators induce rapid degradation of IκB. This activation event is triggered by phosphorylation of IκB by the IκB kinases (IKK).

Phosphorylated IκB undergoes polyubiquitination and is then degraded. Degradation of IκB exposes the NLS on NF-κB, which is then translocated to the nucleus.

Activation of the NF-κB

NF-κB is activated by a range of stimuli, including various pro-inflammatory cytokines, stress signals and pathogens (Ghosh and Karin, 2002). The activation of NF-κB upon bacterial or viral infection is a particularly effective way of initiating an immune response to the infection. Since NF-κB pre-exists in the cell and can be stimulated without new protein synthesis, its activation occurs quickly. The activation of NF-κB promotes then the synthesis of many important immune system regulators. Bacterial and viral products such as LPS and dsRNA as well as the transactivating proteins of Herpes Simplex Virus, human T Cell Leukaemia Virus (HTLV-1), HIV-1 and Hepatitis B Virus activate NF-κB (Kopp and Ghosh, 1995). Regarding the induction of NF-κB by cytokines NF-κB is critically positioned in a network of cytokines involved in immune system function. Several cytokines induce NF-κB and many cytokines are induced by NF-κB thus establishing an autoregulatory feedback loop. The main cytokines known to induce NF-κB are TNF-α and IL-1 (Grilli et al, 1993; Beg et al, 1993).
The NF-κB signalling pathway is schematically presented and shortly described in Figure 1.2. Many pathogens are recognized by specific pattern-recognition receptors (PRRs). The best known PRRs are the Toll-like receptors (TLRs), a group of transmembrane proteins that mediate the activation of intracellular signalling pathways after recognizing extracellular pathogens. TLR2 and TLR4 are essential for the recognition of distinct bacterial cell-wall components like LPS. IL-1 activates NF-κB in a similar manner as LPS because of the homology between the cytoplasmic signalling domains of the IL-1 receptor and the TLRs. TNF-α acts via its own receptors that are present on the surface of a wide range of cells. The most important event in the activation of NF-κB is the phosphorylation of IκBs, which is mediated by IKKs. The IKK complex is a converging point for the activation of NF-κB by a large number of stimuli. IKK mediated phosphorylation and degradation of IκBs promotes rapid translocation of the NF-κB complex into the nucleus, where NF-κB influences the transcription of target genes. NF-κB induces effectors of immune, inflammatory, or acute phase responses. In particular, numerous genes encoding cytokines/growth factors/ chemokines, biological messengers that control and coordinate the function of various cells, are responsive to NF-κB (Table 1.3).

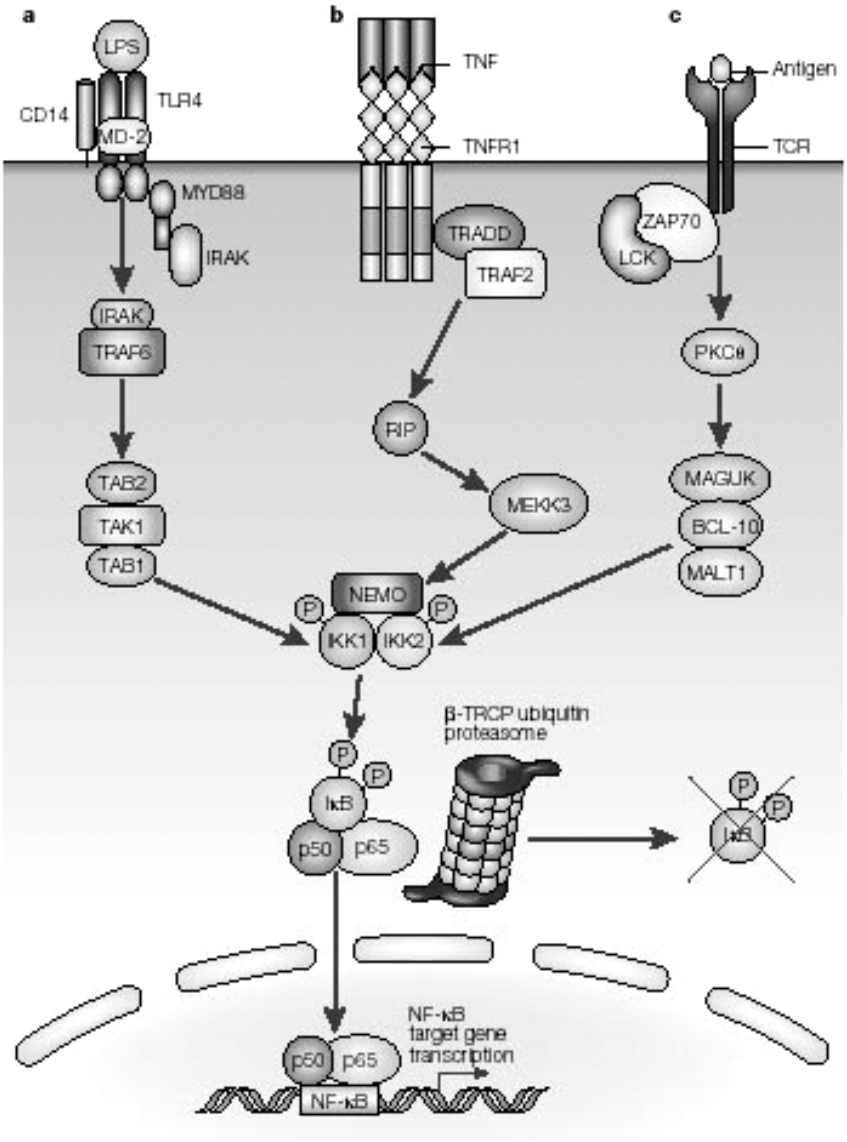

Figure 1.2. NF-κB activation pathways. *NF-κB activity is stimulated by many pathways, including lipopolysaccharide (LPS), tumour-necrosis factor (TNF) and T-cell receptor (TCR) signalling. The inhibitor of NF-κB (IκB) kinase (IKK) complex — composed of the kinases IKK1 and IKK2 and the regulatory subunit NF-κB essential modulator (NEMO; also known as IKKγ) — is a point of convergence for all three signalling pathways.* ***a)*** *LPS binding to Toll-like receptor 4 (TLR4)–CD14–MD-2 complexes activates an intracellular signalling cascade that involves the recruitment of MYD88 (myeloid differentiation primary response gene 88) and IRAK (interleukin-1-receptor associated kinase). Activation of IRAK results in the phosphorylation of TNF-receptor associated factor 6 (TRAF6), which might relay signals through the TAK1–TAB1–TAB2 complex to IKK complexes to activate the NF-κB pathway.* ***b)*** *TNF receptor 1 (TNFR1) engagement by TNF results in receptor trimerization and recruitment of the adaptor protein TRADD, which, in turn, interacts with the carboxyl terminus of TRAF2, an adaptor protein that has additional affinity for various downstream signalling proteins. MAP/ERK kinase kinase 3 (MEKK3) and receptor-interacting serine/threonine kinase (RIP) probably link TNF signalling to IKK activation.* ***c)*** *T-cell stimulation, in response to antigen-presenting cells or anti-TCR-CD3 antibodies results in the rapid translocation of protein kinase Cθ (PKCθ) to the plasma membrane. (Li and Verma, 2002).*

Class of gene	NF-κB dependent gene
Cytokines/growth factors	IL-1α and β IL-2, -3, -6, -8, -12 TNF-α, LT-α, IFN-β G-CSF, M-CSF, GM-CSF
Immunoreceptors	IL-2Rα, TCRα and β, MHC-I, MHC-II
Adhesion molecules	ICAM-1, VCAM-I, MAdCAM-I, E-selectin
Acute phase proteins	Angiotensinogen, SAA, Complement factors B, C3 and C4
Others	iNOS, Vimentin, Cyclin D1, BCL2

Table 1.3. *Selected NF-κB-inducible genes (May and Ghosh, 1998; Siebenlist et al, 1994; Chen and Green, 2004).*

1.2 NF-κB-repressing factor (NRF)

Although NF-κB is kept in its inactive form in the cytoplasm, some level of the NF-κB proteins is present in the nucleus also in the unstimulated state. Further, many events (Figure 1.2) stimulate NF-κB activation, but not all NF-κB-regulated genes are induced by each NF-κB stimulation. Thus, to prevent a constitutive activation of all NF-κB-induced promoters an additional regulation of NF-κB activity should take place. NF-κB-repressing factor (NRF) was initially identified as a constitutively expressed silencer protein that binds to the negative regulatory element (NRE) in the β-interferon (IFN-β) promoter and represses the basal transcription of this gene (Nourbakhsh et al, 1993). Later, additional promoters possessing the NRF binding site were described. NRE-containing promoters in addition to IFN-β include the promoters of IL-8, iNOS genes and HIV and HTLV. The common features exerted by the presently investigated NREs are sequence homology, short length (11-13 bp), distance and position-independent action and specific silencing of NF-κB-binding sequences (Nourbakhsh and Hauser, 1999; Nourbakhsh et al, 2000 and 2001; Feng et al, 2002). A comparison of NREs is summarized in Figure 1.4.

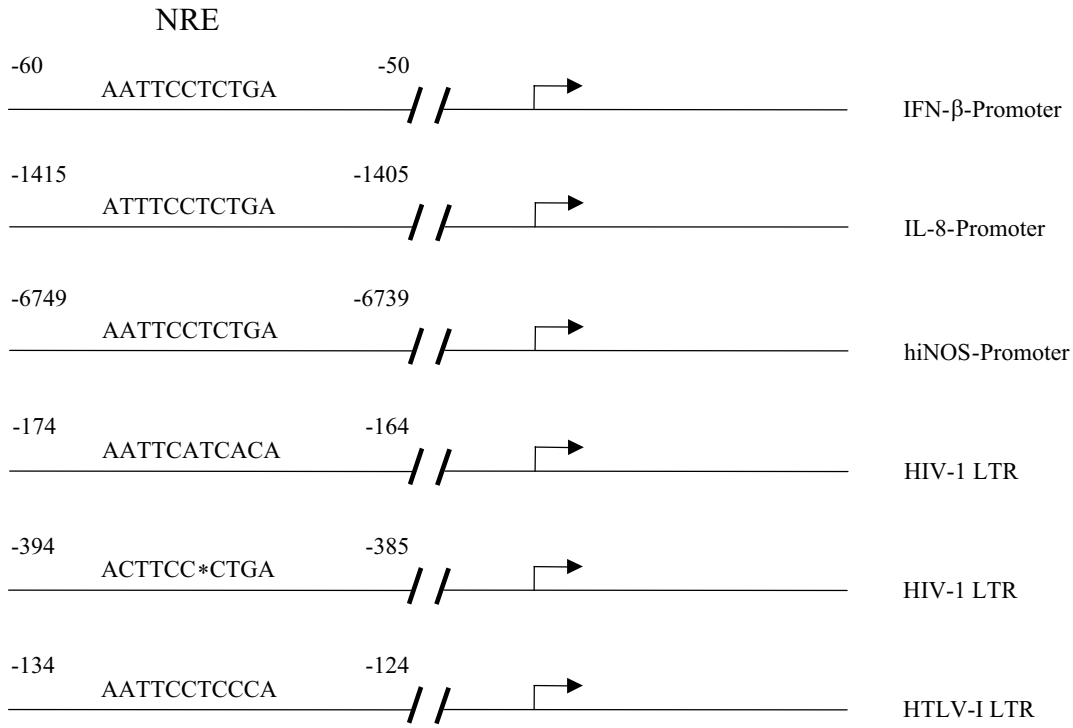

Figure 1.4 *Sequence comparison of the NRE sites in different NF-κB-regulated promoters.*

NRF specifically binds to a NRE and participates in the transcriptional regulation of NF-κB target genes. The cDNA of NRF was cloned. Analysis of the NRF expression pattern shows that NRF is abundant and ubiquitously expressed. NRF mRNA is detected in all tested cell lines and human tissues (Nourbakhsh and Hauser, 1999; Jianfeng et al, 2003).

The NRF mRNA contains a strong IRES element that results in IRES mediated translation (Oumard et al, 2000). EST sequences in public data bases suggest that apart from the NRF mRNA examined earlier, other NRF mRNA species might exist that originate from splicing of a mRNA from a upstream exon. This mRNA lacks the IRES element but leads to a slight extension (18 amino acids) of the N-terminal portion of the NRF protein. Experimentally this mRNA and its protein have not been examined yet. Nevertheless, the biological function of the IRES element is not understood. The data accumulated to date indicate that it confers high translation efficiency which is not perturbed by viral infection and diverse stress responses (Oumard et al, 2000 and data not shown).

Regarding the mechanism of the inhibitory action of the NRF it was shown that NRF at least *in vitro* interacts directly with members of the NF-κB family (Nourbakhsh and Hauser, 1999). Thus, NRF acts through both DNA:protein interactions, as well as protein:protein interactions.

In the chromosomal mapping murine NRF gene was positioned to the A4 region of the X-chromosome (Schwarzer, 2001). The human NRF gene was mapped to human Xq25 (Schwarzer, 2001; Frattini et al, 1997).

The NRF gene contains an open reading frame for a protein of 690 amino acids and is strongly conserved in human, mouse and rat. Within this protein different structural and functional domains were identified (Figure 1.5).

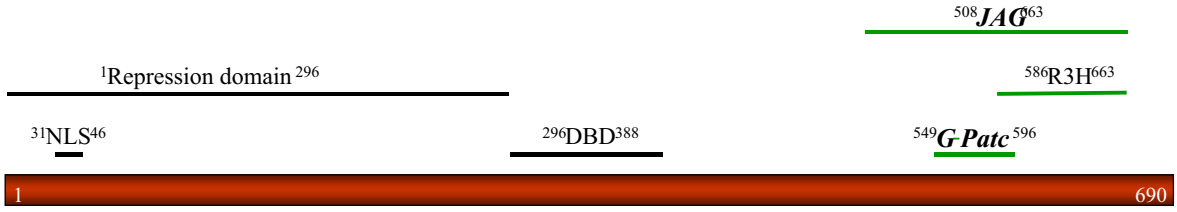

Figure 1.5. *Schematic structure of the NRF protein (according to Niedick et al, 2004).*

The DNA (NRE)-binding domain and a domain responsible for constitutive NF-κB repression as well as a nuclear localization signal (NLS) were located within the N-terminal part of the NRF protein (amino acids 1-388). This is the size of the originally described NRF protein. Sequence characterization and blast analysis revealed the existence of a C-terminal part of the NRF protein (Niedick et al, 2004). This contains domains including a glycine rich nucleic binding domain (G-patch) and a single-stranded nucleic acids-binding domain (R3H). These two domains represent potential nucleic-acid binding sites. The G-patch domain exists in a number of putative RNA-binding proteins involved in DNA-damage repair and tumor supression. Additional dsRNA-binding domains (dsRBD) are found dispersed throughout the C-terminal half of the NRF protein (from amino acid 362 to amino acid 550). Although a precise definition of the RBD could not be achieved, a binding of the NRF protein to dsRNA was clearly confirmed (Niedick et al, 2004).

The subcellular localization and mobility of the NRF protein was studied (Niedick et al, 2004). NRF is highly enriched in nucleoli and only little protein is found in the nucleoplasm and cytoplasm. It was found that the mobility of NRF located in nucleoli is much lower compared to the mobility of nucleoplasmic and cytoplasmic NRF. The dynamic movement of a protein in the cell depends not only on its size and shape but also on interactions with other factors. Thus, it was suggested that the NRF protein is somehow fixed in the nucleolus. This could be due to a direct or indirect binding to nucleolar structures.

NRF is a repressor protein that is thought to be mainly active in the non-induced state of the cells. It is possible that NRF is involved in the repression of transcription of nucleolar genes.

This would suggest that the NRF regulated genes are kept in nucleoli in the inactive state but are released to the nucleoplasm upon transcriptional activation. This hypothesis was not examined yet.

The promoters in which the inhibitory action of the NRF were best studied are IFN-β (Nourbakhsh and Hauser, 1999) and iNOS (Feng et al, 2002). The IFN-β promoter was shown to be constitutively repressed by a negative regulatory domain (NRD). The NRD was defined by the successive deletion of the 5' regulatory region of the IFN-β gene which led to activation of the IFN-β promoter (Goodbourn and Maniatis, 1988). Within the NRD a negative regulatory element NRE was identified (Nourbakhsh et al, 1993). Later, the NRF protein that binds to the NRE was identified. Deletion or distinct point mutations of the NRE as well as inhibition of the NRF were shown *in vitro* to affect the constitutive silencing of the IFN-β gene expression.

1.3 NRF target genes

Interferon β (IFN-β)

IFN-β belongs to the family of type I interferons. Interferons (IFN) were discovered as antiviral agents during studies on viral interference. The interferon system includes cells that synthesize IFN and the cells that respond to IFN by establishing an antiviral state. IFN proteins display autocrine as well as paracrine activities. The IFN response represents an early host defense and is thus part of the innate immune system. The IFN-β gene is absolutely silent, but can be rapidly activated in nearly all differentiated cells. Viruses and dsRNA are potent inducers of the IFN-β gene expression. Macrophages produce IFN in response to exposure to microbial pathogens or to microbial products like LPS or bacterial DNA (Bogdan et al, 2000). Many agents, such as the biological response modifiers IL-1, TNF-α and PDGF, are also able to induce the expression of IFN-β, but to a lower extend. IFN-β possesses a wide range of biological activities in addition to the characteristic antiviral activity including regulation of cell growth, differentiation and apoptosis. IFN-β acts through binding to cell surface interferon type I receptors and subsequent activation of the Jak/STAT signalling pathway (Samuel et al, 2001). Among the genes induced by the Jak/STAT pathway are those of the IRF family. Cooperation of the IRF, STAT and NF-κB transcription factors then results in the induction of a network of antiviral genes, including the well characterized dsRNA

activated kinase PKR, 2'-5'-oligoadenylate synthase and the Mx proteins that interfere with viral transcription and translation processes.

The highly specific activation of the IFN-β promoter in response to virus infection requires an overlapping set of the regulatory elements termed positive regulatory domains (PRDI-PRDIV). PRDII, PRDI-III and PRDIV are recognized by the transcription factors NF-κB (p50/p65), IRF-1 and ATF-2/c-Jun respectively (Thanos and Maniatis, 1995). Maximal activation of the IFN-β promoter requires cooperative binding of these transcription factors together with the high mobility group protein HMGI(Y) which form a multicomponent complex, termed the enhanceosome (Figure 1.6).

LPS induced activation of the IFN-β gene is mediated by TLR4 and leads to the activation of NF-κB and IRF-3 (Yamamoto et al, 2004).

In virus-induced cells, three negative regulatory proteins have been identified modulating the activity of PRDs in the IFN-β promoter. IRF-2 and PRDI-BF1/Blimp-1 were described to bind to PRDI (Harada et al, 1989; Keller and Maniatis, 1991), while PRDII-BF1 was isolated by binding to PRDII (Fan and Maniatis, 1989). The virus-inducible expression kinetics of these proteins have established that they are involved in the mechanism of the post-induction silencing.

In addition to gene regulatory proteins, chromatin remodeling plays an important role in regulation of gene expression. The fundamental structural units of chromatin, the nucleosomes, are composed of an octet of histones. The amino-terminus of histone polypeptides is subject to different modifications, including phosphorylation, acetylation, methylation and ubiquitylation. These modifications affect chromatin structure and contribute to the regulation of transcription (Chen and Greene, 2004). It was demonstrated that histone acetylation is important regulatory mechanism in the IFN-β and the IL-4 promoters (Shestakova et al, 2001; Valapour et al, 2002). In general, acetylated histone tails are found in transcriptionally active segments of chromatin, whereas deacetylated histones as well as their methylated forms accumulate in transcriptionally repressed regions of chromatin (Imhof and Wolffe, 1998).

The NRE is required for position-independent silencing of the NF-κB site of the IFN-β promoter. It was shown that *in vitro* NRF is permanently bound to the NRE and represses constitutive activation of the IFN-β promoter. In cell culture experiments the expression of the NRF antisense RNA releases the endogenous IFN-β gene transcription (Nourbakhsh and Hauser, 1999).

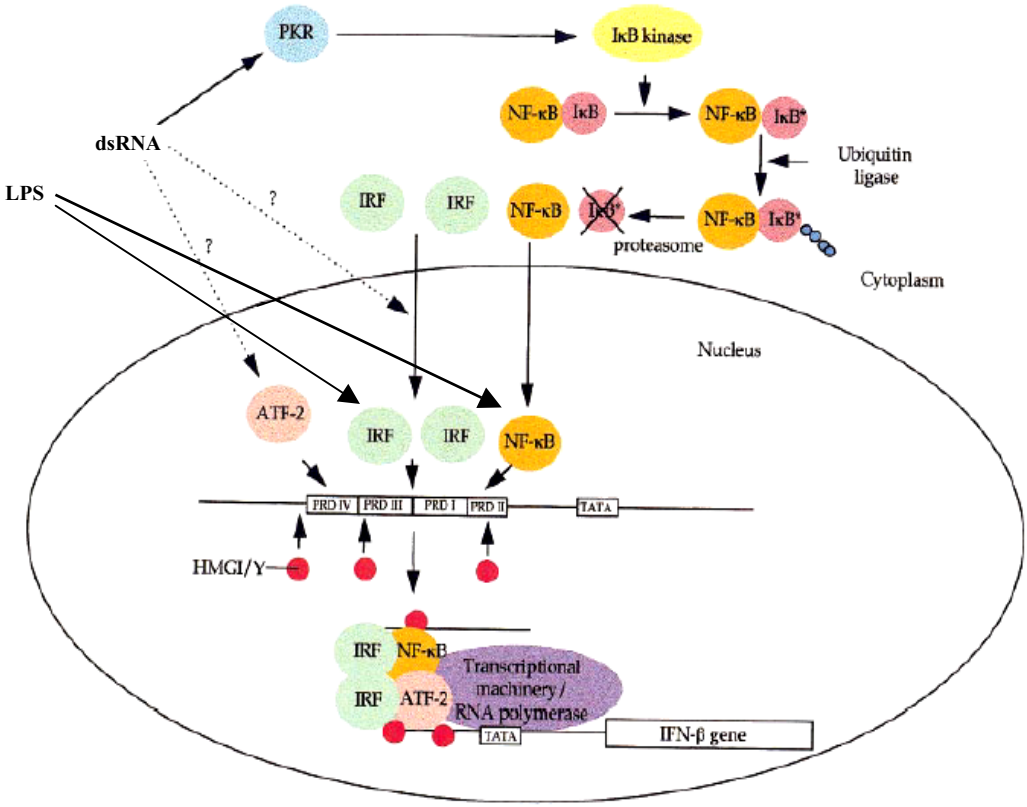

Figure 1.6. *Transcriptional induction of the IFN-β gene. Virus replication gives rise to dsRNA, which is able to activate PKR and perhaps additional cellular kinases. PKR in turn activates the IkB kinase and indirectly leads to the activation of the immunomodulatory transcription factor NF-kB. Together with ATF-2 and a member(s) of the IRF family, NF-kB assembles on the IFN-β promoter with the help of several copies of the accessory factor HMG-I/Y to form a multifactorial complex called the'enhanceasome'. Components of the enhanceasome make contacts with factors that are part of the basal transcriptional machinery and, by stabilizing interactions with this machinery and causing a local 'remodelling ' of the chromatin, recruit RNA polymerase II to the promoter to bring about transcription of the IFN-β gene. Alternatively, activation of TLR4 by LPS induces a signalling cascade leading to activation of IRF-3. Activated IRF-3 forms a homodimer and translocates into the nucleus to switch on the induction of IFN-β.*

Inducible nitric-oxide synthase (iNOS)

The inducible isoform of nitric-oxide synthase catalyses the production of nitric oxide (NO). The NO is generated from the iNOS substrate L-arginin by oxidation of one of the guanidino nitrogens. NO is a free radical that acts as a signaling agent and a cytotoxic molecule and has microbicidal, antiviral, antiparasital and antitumoral activity (Kleinert et al, 2003).

Like IFN-β, iNOS gene is silent in the unstimulated state, but can be induced by different agents. Murine cells express iNOS in response to bacterial lipopolysaccharide (LPS), stimulatory cytokines such as IFN-γ, IL-1, IL-6, TNF-α and some other. In contrast, the majority of human cells require a complex cytokine combination including IFN-γ, IL-1 and TNF-α for iNOS induction (Taylor et al, 1998). The induction of iNOS by LPS occurs through the activation of a TLR4-dependent signaling cascade, resulting in the activation of NF-κB. The sequences of the iNOS promoters in various species including humans and mice exhibit homologies to binding sites for numerous transcription factors such as AP-1, GATA, IRF-1, NF-1, Oct-1, Stat1 and NF-κB (Kleinert et al, 2003). iNOS activity is regulated predominantly at the transcriptional level. It was shown that NF-κB and Stat1 play a key role in this regulation (Taylor et al, 1998; Ganster et al, 2001). Upstream to NF-κB binding site a NRE-related element was identified in human iNOS promoter (Feng et al, 2002). It was also confirmed that in cell culture experiments the NRF protein is involved in constitutive silencing of the human iNOS gene by binding to a NRE.

IL-8

Interleukin-8 (IL-8) is a member of the family of chemokines that acts as mediator of acute inflammation. IL-8 synthesis can be induced by pro-inflammatory cytokines such as IL-1 or TNF or as a direct consequence of contact with pathogens like bacteria, viruses and stress agents. NF-κB plays a key role in the transcriptional regulation of IL-8 gene. By sequence comparison with the IFN-β promoter, a NRE was found in the IL-8 promoter. It was also shown that NRF binds to the IL-8 promoter NRE. But in contrast to the its role in IFN-β and iNOS promoters, in IL-8 transcription NRF was found to have a dual role. In the absence of stimulation, NRF is involved in transcriptional silencing, but, in the presence of IL-1, it is required for full induction of the IL-8 promoter (Nourbakhsh et al, 2001).

2. Results

Objectives

Transcriptional regulation of most cytokine genes is characterized by strict constitutive repression and stimuli-specific activation. The molecular mechanism of this constitutive repression is still poorly understood. The recently identified NRF protein (NF-κB repressing factor) was proposed to act as inhibitor in some NF-κB regulated promoters. NRF was shown to have inhibitory effects on IFN-β (Nourbakhsh and Hauser, 1999), human iNOS (Feng et al, 2002) and IL-8 (Nourbakhsh et al, 2001) promoters. These studies were carried out on the model of *in vitro* cultured cell lines. With these methods a) characterization of the protein function is restricted to a certain cell line and b) cannot enlight interactions of the studied protein with other components of the biological system. The developing of gene targeting techniques allows the analysis of diverse aspects of gene function in the context of the whole organism. Gene targeting involves the inactivation of a given gene in the genome of embryonic stem (ES) cells by homologous recombination. ES cells retain their totipotency and can participate in the generation of cell lineages of the mouse. Transfer of mutant ES cells into mouse embryos thus allows the transmission of the mutation into the mouse germ line and generation of animal carrying the mutated gene. Such mutant mice represent a tool to investigate the functions of individual genes in the complex biological system. **Aim of this work was to understand the *in vivo* function of NRF on the model of NRF-KO mice and to study the consequences of NRF deletion in the mutant organism.** Because of the binding of NRF on various promoters and its inhibitory role it was hypothesized that 1) NRF has co-functional (inhibitory and stimulatory) effects on different NF-κB regulated promoters 2) NRF deficiency can lead to spontaneous expression of certain genes, as for example IFN-β or iNOS. Such dysregulation of cytokine expression should result in an abnormal phenotype of NRF-deficient mice and lead to disturbance of immune defense. In the first part of the work presented here the NRF-transgenic mice were created and characterized. The second part deals with the effects of NRF protein absence on the molecular level. For this purpose different cell types lacking NRF expression were established and analysed.

2.1 Creation of the NRF-flox mice

In previous work it was shown (Schwarzer M, 2001), that NRF is expressed already at early stages of mouse development. For this reason it was hypothesized that a complete NRF

knock-out should lead to lethality during embryogenesis. A variety of strategies have been developed to bypass this problem. Of those, the most efficient ones uses the Cre recombinase system (Nagy, 2000). The Cre recombinase enzyme of bacteriophage P1 catalyzes recombination between two 34 base pair loxP sites (Abremski et al., 1983). If the loxP sites are placed in the same orientation on a strand of DNA, the outcome of this enzymatic reaction is the excision of the DNA fragment between the loxP sites. Placing the loxP sites itself does in most cases not affect gene expression so that the deletion does not appear until Cre-mediated excision has occured.

For this purpose a targeting vector was constructed (Schwarzer, 2001). This vector carries the endogenous NRF gene, in which part of the second exon is flanked by two loxP sites, with insertion of an artificial STOP codon, so that in mice carrying this cassette a short form of NRF should be expressed. This short form has been shown to carry the DNA-binding and the NF-κB interacting domains (Nourbakhsh and Hauser, 1999). Following expression of Cre-recombinase the second exon will be excised resulting in a nearly complete deletion of the NRF protein (Figure 2.1). In addition, the targeting vector contains the gene for neomycin resistance allowing selection of transfected embryonic stem (ES) cells.

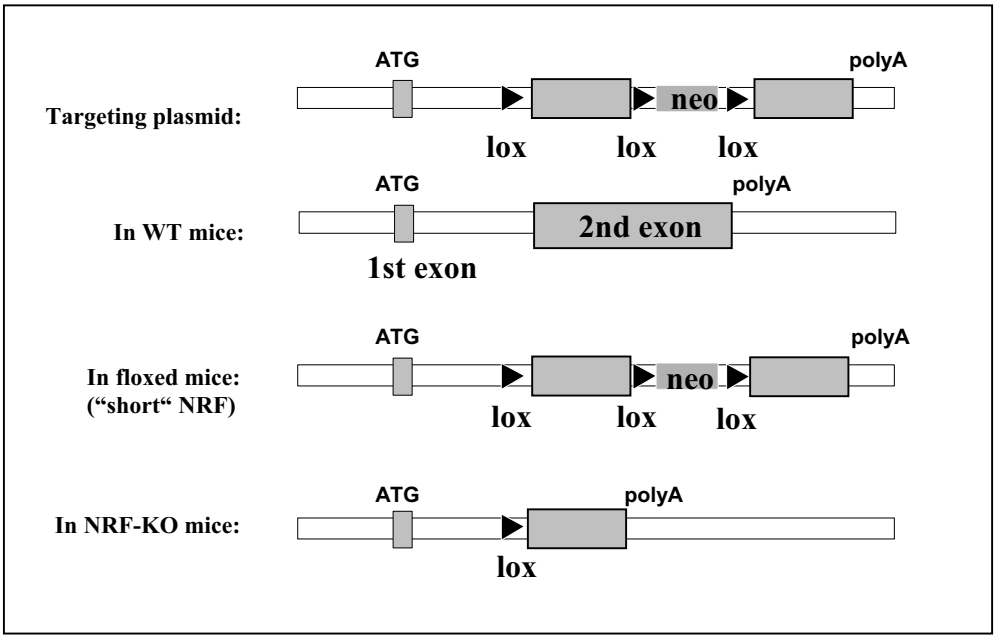

Figure 2.1. *Schematic structure of the targeting vector and the genomic modification in transgenic mice. In WT mice the NRF protein is expressed (1-690 amino acids), while gene targeting is associated with the expression of a "short" version of the protein (1-388 amino acids) in floxed animals or lack of the protein in NRF-KO mice (1-38 amino acids).*

The targeting plasmid was electroporated into ES cells and after about 10 days of neomycin selection the resistant clones were tested by PCR and Southern Blot analysis. For the PCR the

primers were designed in such a way that the upstream one (neo) is neomycin gene specific and locates inside the exchanged DNA cassette, while the downstream primer (3'-out) hybridizes to the endogenous part of NRF gene (Figure 2.2). With these primers one can detect clones that have undergone recombination and contain the gene for neomycin resistance in their genome. From the tested ES cell clones 3 (5A2, 6A5 and 2B2) were found to be recombinant (Figure 2.3).

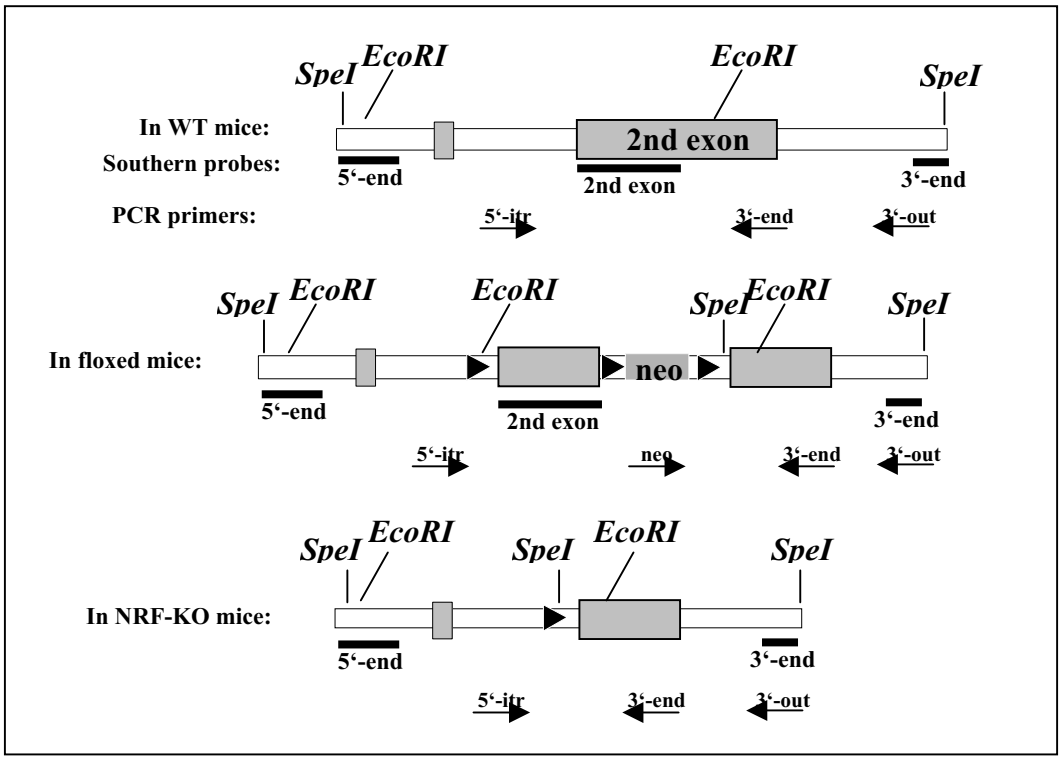

Figure 2.2. *Schematic presentation the orientation and location ofprimers (arrows) and of Southern analysis probes (bars) used in this study. Important restriction enzyme sites are also indicated.*

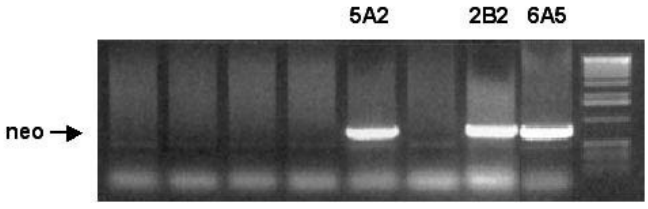

Figure 2.3. *Screening of targeted ES cells clones. Genomic DNA was isolated from the targeted one-cell clones of ES cells and tested by PCR with neo-specific primers.*

To confirm this data and to show specificity of homologous recombination, positive clones were tested with Southern Blotting using three different probes: 5'-specific, 2nd-exon specific and 3'-specific probes (Figure 2.2). Transgene loxP-NRF produces a 6.2 kb *EcoRI* fragment when hybridized with 5'-specific probe, a 4.8 kb *SpeI* fragment with the 3'-specific probe and an 8.7 kb *SpeI* fragment when hybridized with the 2nd-exon-specific probe. An endogenous

allele should give the following fragments: a 7.5 kb *EcoRI* fragment when hybridized with the 5'-end probe; a 13.6 kb *SpeI* fragment with the 3'-end specific probe and a 13.6 kb *SpeI* fragment with the 2nd-exon probe. Fragments of different size can be clearly distinguished on the Southern blots. All three tested cell clones show the same pattern (Figure 2.4) that correlates with the pattern expected in the case of successful homologous recombination. Thus these clones were defined as NRF-floxed.

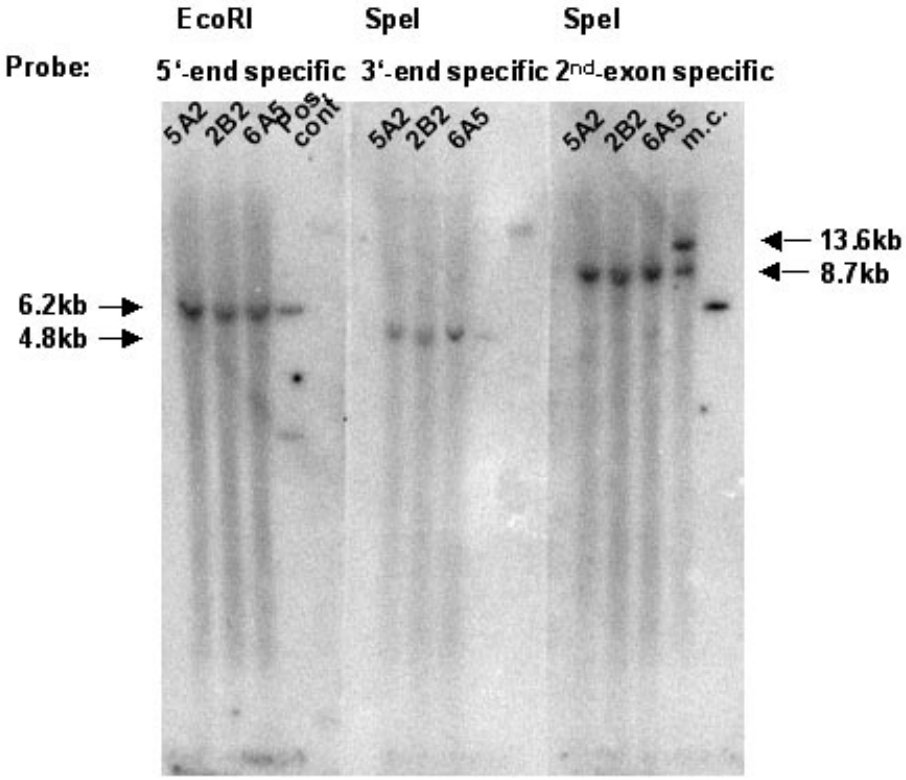

Figure 2.4. *Southern blot analysis to confirm homologous recombination in the PCR positive clones 5A2, 2B2 and 6A5. Genomic DNA was digested with EcoRI or SpeI and hybridized with 5'-end, 3'-end or cDNA specific probes as indicated. Pos.cont.=positive control and represents digested plasmid DNA with only NRF-flox possible variant of the gene. M.C=mixed clones: DNA isolated from the mixed NRF-flox and WT cell populations produce therefore both the transgene and the endogenous fragments.*

Based on morphological characteristics of the cells, clone 2B2 was chosen for blastocyst injection. These NRF-flox ES cells were injected into blastocysts and implanted into pseudopregnant recipient mice. As a result, chimeric mice were born. From 5 chimeric mice we received 3 males and 2 females with different level of chimerism (40-95%), but only one male was able to transmit the NRF transgene into the germ line. This chimeric male was crossed with C57BL/6 wild type mice to get a heterozygous NRF-flox female or a homozygous NRF-flox male (the NRF gene is linked to the X-chromosome). The genotype of all offsprings from this cross was analysed. In the F1 generation mice the NRF transgene can

be detected by PCR and Southern Blot analysis. For PCR neomycin resistance gene specific primers were used, which revealed the neo-containing flanked form of the NRF gene (data not shown). For Southern Blot analysis the NRF 2nd-exon specific probe was used (Figure 2.5). In the case of heterozygous females two bands were detectable, correlating with the endogenous wild type (WT) and the introduced 2B2 or NRF-flox allele. The colony of transgenic mice carrying the NRF-flox allele was then established by further breeding of F1 generation mice.

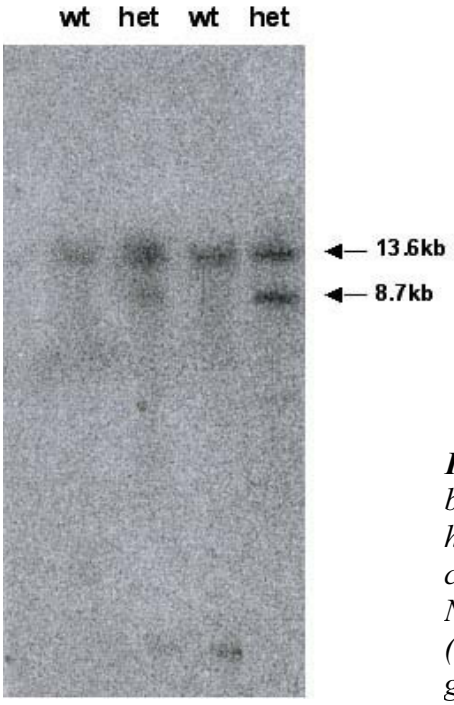

Figure 2.5. *Genotyping of F1 generation mice by Southern bloting. Genomic DNA was digested with SpeI and hybridized with an NRF cDNA specific probe. The samples containing both the endogenous WT (13.6 kb) and the NRF-flox (8.7 kb) allele were defined as heterozygous (het). WT mice express only the endogenous form of NRF gene.*

2.2 Creation of the NRF-KO mice

In order to get NRF-KO mice NRF-flox mice have to be crossed to Cre-recombinase expressing mice. The specificity of gene inactivation depends on the expression pattern of Cre recombinase and its regulation. Using time and tissue specific promoters driving Cre expression, the flanked gene can be specifically deleted at different stages of the mouse development or in the specific tissue or cell types. First, we were interested in effects of a complete deletion of the NRF gene and so we used a so called deletor strain of Cre-expressing mice. In these mice Cre-recombinase is expressed during early embryogenesis, so that excision of the flanked allele occurs before organs and tissues are formed, resulting in a complete deletion of the gene. For this work K14-Cre mice were used (Hafner et al, 2004). K14-Cre mice express Cre under the control of the keratin 14 promoter. Because of the activation of the K14 promoter specifically in the oocyte, when Cre is transmitted by K14-Cre females mated to NRF-flanked males, even offsprings that do not inherit Cre, delete the target

gene. Figure 2.6 shows the expected pattern of genotypes produced from the cross of a heterozygous Cre expressing female with a male carrying the NRF flanked allele.

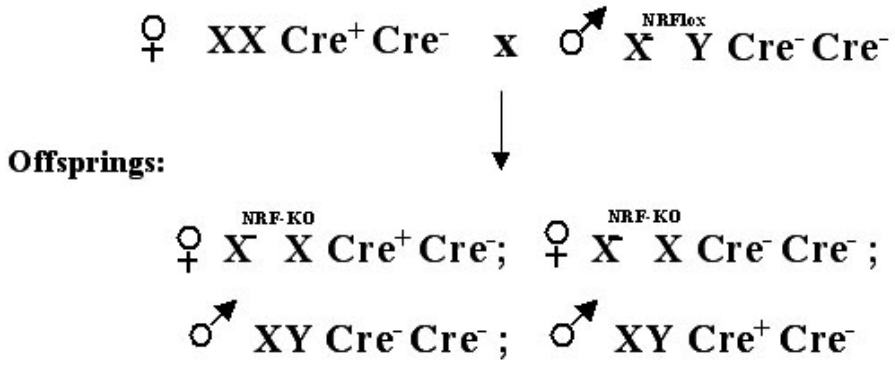

Figure 2.6. *Breeding scheme: crossing between a heterozygous Cre female and a NRF-flox male. The excision takes place in all zygotes inheriting the flanked allele, also when the offspring does not inherit Cre.*

According to the rules of heredity of X-linked genes, females get one of their X chromosomes from their father; here it is carrying the NRF flanked allele. In all female zygotes the NRF gene will be immediately excised by the Cre recombinase. This occurs independently from inheritance of the Cre gene because of the expression of the Cre protein already in the outgoing oocyte. Thus, all female offsprings from this cross will be heterozygous for the NRF-KO allele. To get homozygous animals series of additional crosses were performed. The mice were identified by PCR analysis of DNA isolated from tail biopsy samples. The primer pair was designed in the way that different size bands corresponding to WT, NRF flox and NRF-KO alleles can be distinguished (location and orientation of primers shown on the Figure 2.2). Figure 2.7 shows a representative PCR gel.

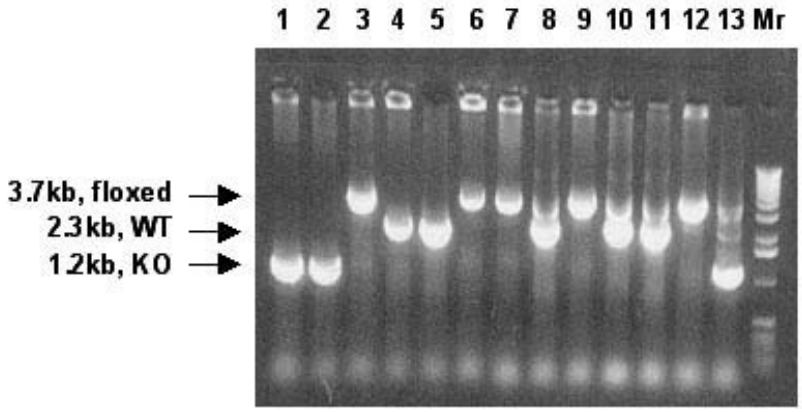

Figure 2.7. *Genotyping of mice. PCR analysis of 13 mice using 5'-itr and 3'-end specific primers. The WT allele gives a 2.3 kb fragment, the flanked form of NRF gene –a 3.7 kb band, excision of the gene results in a 1.2 kb fragment.*

According to these results mice 1 and 2 contain only the NRF-KO allele, mice 4 and 5 are WT, mice 3, 6, 7, 9 and 12 are NRF flox, mice 8, 10, 11 have both WT and flanked alleles and are therefore heterozygous, and mouse number 13 is KO heterozygous. To confirm the lack of the NRF gene, DNA of KO mice was also analysed by Southern bloting. The DNA blot was first hybridized with a NRF 2nd exon specific probe (Figure 2.8, A). In lanes corresponding to the KO samples we could not detect any specific band. In contrast, in control samples the different size bands are visible, indicating WT or NRF flanked alleles. A second hybridization of the same blot with a 5' specific probe revealed smaller DNA fragment in samples from NRF-KO mice, confirming the NRF gene deletion in these samples (Figure 2.8, B).

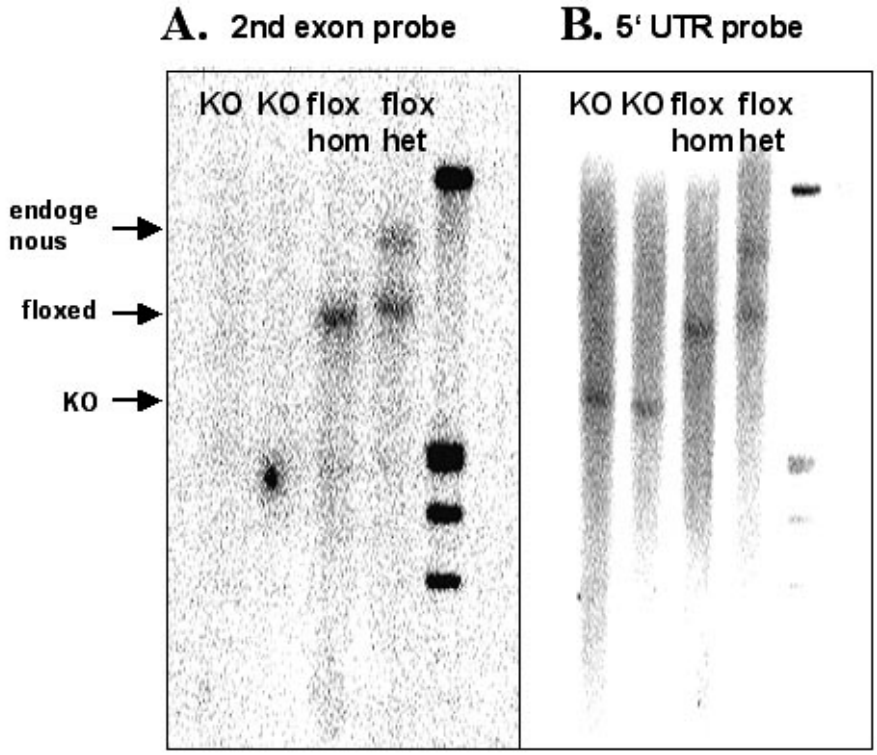

Figure 2.8. *Southern blot analysis of DNA from transgenic mice. Genomic DNA isolated from tail byopsies was digested with EcoRI and hybridized with the 2nd exon (A) and the 5'-end (B) specific probes as indicated. Hom=homozygous, het=heterozygous mice.*

A primary characterization of homozygous NRF-KO mice revealed that the lack of the NRF gene is compatible with life and does not cause any visible pathologic effect.

2.3 Establishment of cells lacking NRF expression

The studies on effects resulting from a lack of the NRF protein on the molecular level were carried out with cell cultures *in vitro,* because these effects can much better be studied in

homogenous population of cells. Since NRF can bind to different promoters, which are active in different cell types, molecular effects of NRF deletion were studied in specifically differentiated cells. For this purpose the following NRF-KO cells were established: ES cells, *in vitro* differentiated endothelial cells, cell cultures of primary fibroblasts and macrophages.

In vitro excision of the NRF gene in ES cells

A method for the creation of mice expressing NRF-floxed allele from the appropriate ES cells with subsequent excision of the gene by breeding with a Cre-expressing mice requires a long-term protocol. For this reason it was decided to use a short-time protocol and establish a line of ES cells lacking the NRF gene by using a method of *in vitro* excision of the NRF gene in ES cells. An additional reason for the application of this method was that the NRF gene is linked to the X-chromosome and ES cells expressing the NRF-floxed allele are masculine and have only one X chromosome per cell. Thus excision of the NRF-floxed allele results directly in NRF-KO cells. In addition, functionality of Cre-mediated NRF excision can be tested *in vitro*. For this purpose the Cre-recombinase was expressed in NRF-flox ES cells (clone 2B2). A plasmid encoding the GFP-Cre fusion protein (Gagneten et al, 1997) was introduced into ES cells by electroporation. The cells expressing Cre-recombinase also produced GFP and could therefore be selected. We isolated the GFP expressing cells by Flow cytometric cell sorting and received about 5% positive cells (Figure 2.9).

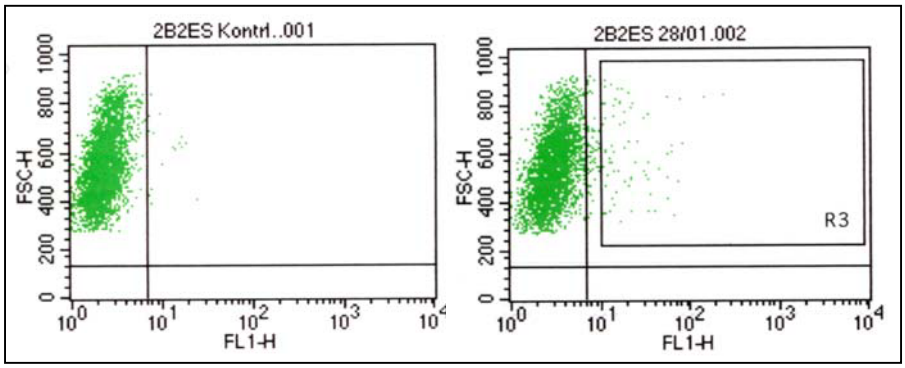

Figure 2.9. *Sorting of GFP-Cre transfected ES cells. NRF-flox ES cells were electroporated with GFP-Cre DNA. 48 hours later GFP positive cells were sorted by Flow cytometry cell sorting.*

The sorted cells were cultured further and single cell clones were isolated from this culture and tested for efficiency of Cre-mediated excision. When excision had occurred and the floxed cassette was deleted, one should detect a genomic pattern corresponding to that of the NRF-KO situation (see Figure 2.1). The cell clones were analysed by PCR and Southern Blot

analysis. Figure 2.10, A shows a representative gel of the PCR products. With the 5'-UTR and the 3'-end primer pair both NRF-flox and NRF-KO alleles could be detected (Figure 2.2). The NRF-flox allele gives a 3,7 kb band while the PCR product corresponding to the NRF deletion has only 1,2 kb. Having this in mind clones 1, 2, 3 and 5 harbor the NRF-KO allele, while the clone number 4 was not a pure clone or a clone without deletion. A second PCR was performed using neomycin gene specific primers (Figure 2.2). If excision of the NRF-flox cassette does not occur, the PCR product should be detectable. As it can be seen in Figure 2.10, B in DNA from the clones 4 and 5 one can detect the gene for neomycin resistance. The clones 1, 2 and 3 are negative for the neomycin gene. This correlates with the deletion of the floxed cassette. Clone number 5 harbors both, the NRF-flox and the deleted allele. This can be explained by a mixed cell population.

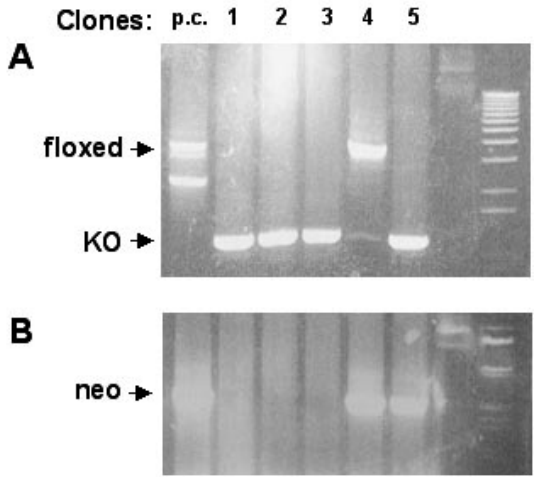

Figure 2.10. *PCR analysis of DNA from GFP-Cre transfected ES clones. A: with NRF-specific primers; B: with neo-specific primers. p.c.=positive control for NRF-flox gene.*

To confirm the PCR results and to exclude unspecific integration of the excized DNA fragment, the clones were also tested by Southern Blot analysis. The DNA blot was hybridized first with a 5'-end specific probe, then stripped and re-hybridised with the NRF 2nd-exon specific probe (Figure 2.11). Hybridisation with the 5'-end specific probe gives a 5.7 kb fragment in clones 1, 2 and 3, that is smaller as that found in other clones or in the control DNA (8.7 kb) and correlates with an NRF gene deletion (Figure 2.11, A). Successful NRF gene excision in clones 1, 2 and 3 is confirmed by the second hybridization of the DNA blot with NRF 2nd-exon specific probe. These clones lack the full NRF gene in their genome (as a result of Cre-mediated excision) since no bands could be detected after hybridisation of DNA with the NRF 2nd-exon specific probe (Figure 2.11, B). The NRF flox allele gives a 8.7 kb fragment.

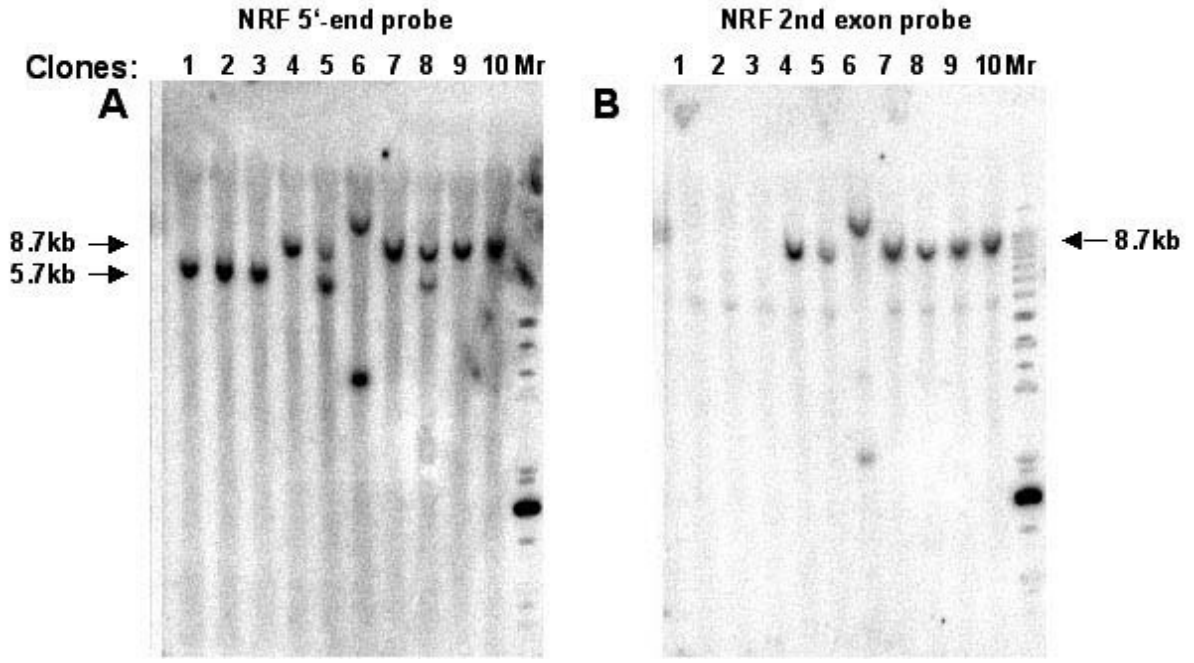

Figure 2.11. *Southern blot analysis to confirm Cre-mediated excision of NRF gene in ES cell clones. Genomic DNA isolated from ES cels clones was digested with SpeI and hybridized with a 5'-end (A) and a 2nd exon specific (B) probe respectevly.*

Putting all this data together we conclude that the NRF-flox cassette was successfully excized after expression of Cre-recombinase in the ES cell clones number 1, 2 and 3. These cells were therefore defined as NRF-KO. The morphology of NRF-KO ES cells could not be distinguished from WT cells by visual inspectation. Further, these cells show no difference with respect to the proliferation rate.

In vitro differentiation of ES cells

The next step in this study was to create cell lines of differentiated cells expressing mutant forms of NRF. ES cells could be differentiated *in vitro* in number of cell types, as for example, muscle, endothelial, neural and some other types. To answer the questions of this study it was decided to differentiate NRF-deficient ES cells into endothelial cells. This is of advantage since endothelial cells are able to express IFN-β as well as iNOS, the key genes shown to be regulated by NRF. The problem of all ES cell differentiation methods is the heterogeneity of the cell population. To overcome this we decided to use a recently published endothelial cells differentiation protocol (Balconi et al, 2000), that leads to an almost pure homogenous population of endothelial cells. The method is based on the use of an endothelial cell specific cocktail composed of growth and differentiation factors. This is used during the differentiation of ES cells and is followed by a selective immortalization of endothelial cells

with Polyoma virus middle T antigen (PymT). PymT was described as immortalization agent the exclusively immortalizes endothelial cells (Williams et al, 1988) because of its ability to activate cell signalling that is crucial for proliferation of endothelial cells.

NRF-KO and WT ES cells were differentiated *in vitro* following a protocol, which is outlined in the Figure 2.12.

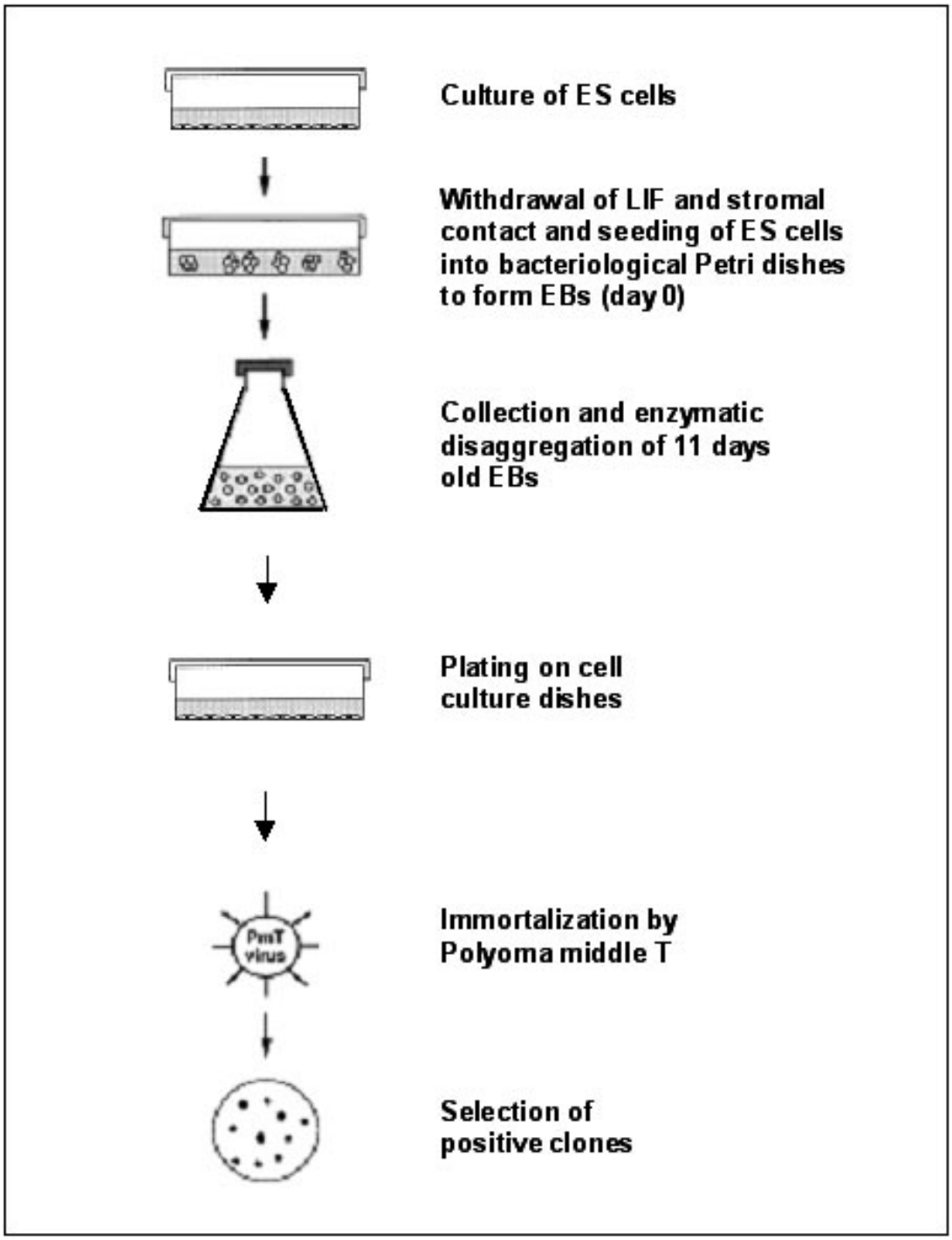

Figure 2.12. *Schematic representation of procedure to differentiate endothelial cells from ES cells. To induce ES differentiation, LIF and feeder cell were removed, and ES cells were cultured in the presence of a cocktail of endothelial cell growth factors. Cells formed embryoid bodies, which were then collected and disagregated. Endothelial cells were developed by direct infection with PymT and selection of puromycin resistant clones.*

ES cells were allowed to differentiate to endothelium by culturing the cells in suspension in the presence of the mixture of growth factors. Under this condition, ES cells organize into embryonic bodies (EBs). 11 days old EBs were then disaggregated, the cell mixture plated on the cell culture dishes and infected with a retrovirus encoding PymT. Endothelial cells expressing PymT were selected with puromycin. Non-endothelial cells were lost during the cultivation since they lost their proliferation potential. At about the 28th day of cultivation puromycin resistant clones of proliferating cells were received, that had a differentiated morphology (Figure 2.13). The presence of PymT gene in the genome was confirmed by PCR (Figure 2.14).

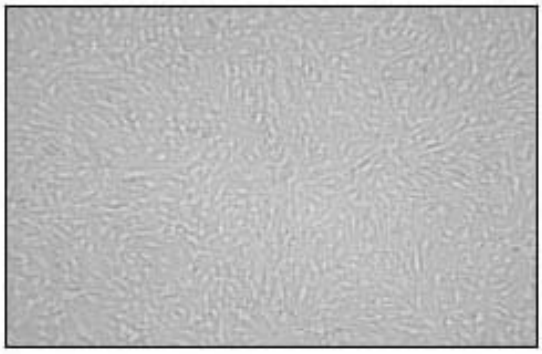

Figure 2.13. *In vitro differentiated endothelial cells. ES cells were differentiated following described protocol. Phase-contrast microscopy of represantative field is shown (x10).*

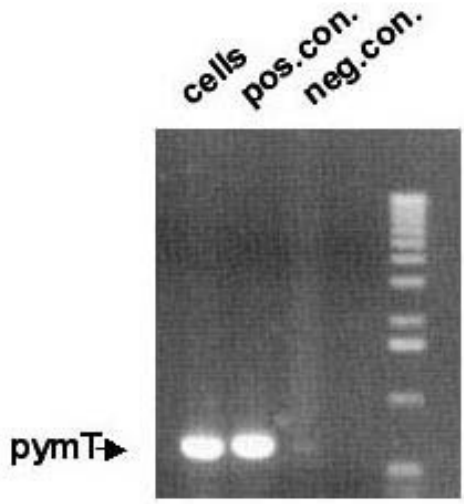

Figure 2.14*. PCR analysis of PymT expressing virus infected and in vitro differentiated endothelial cells. PymT-specific primers were used. Pos.con.=positive control: DNA of virus producer cells; neg .con.=negative control: DNA of non-infected cells.*

To show endothelial properties of those *in vitro* differentiated cells the expression of a typical cell surface marker, expressed by endothelial cells was checked: CD34. The cells were hybridised with FITC-conjugated anti-CD34 antibodies and measured by FACS (Figure 2.15).

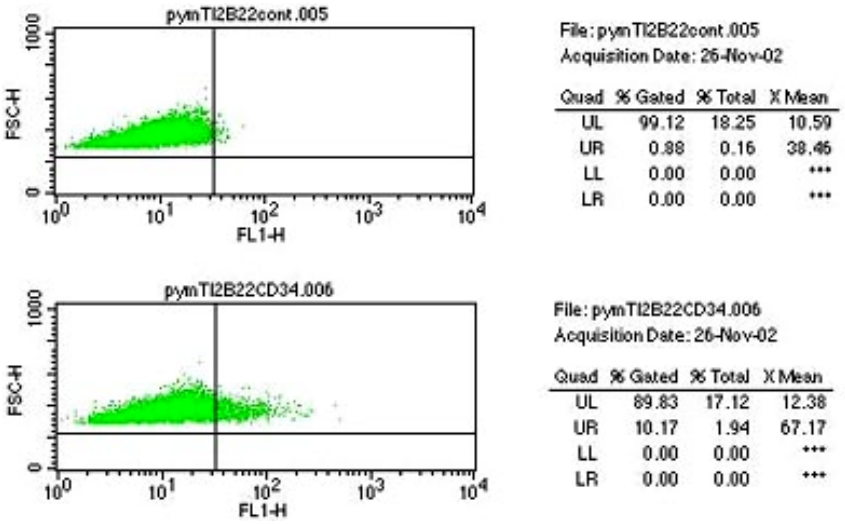

Figure 2.15. *FACS analysis of in vitro differentiated endothelial cells. The cells were stained with FITC-conjugated anti-CD34 antibodies and measured (lower pannel). The upper pannel shows fluorescence level of the control not-stained cells.*

As it can be seen, about 10% of the cells express the CD34 endothelial marker. The low representation of this marker can be explained by a inhomogenous population of cells. This could be due to a relative early stage of this cell culture (pasage 4) at which other non-endothelial cells are still present, indicating that the cell population is not homogenous. It is assumed that those are eliminated during further culture. We conclude that by this *in vitro* differentiation and immortalization of ES cells endothelial cells were obtained and can be used for specific expression analysis.

Cell cultures of primary cells

Primary fibroblasts were isolated from the skin of newborn 2-3 days old mice. These cells have a high proliferation potential, can be propagated in culture beyond passage 8, are easy to keep, give rise to homogenous cultures and represent therefore an excellent model for *in vitro* studies. The primary skin fibroblasts isolated from WT and NRF-KO mice were morphologically indistinguishable and had similar proliferation rates.

Resident peritoneal macrophages were freshly isolated for each experiment from the peritoneal space of WT and NRF-KO mice. These cells play a major role during immune response and inflammation process. Macrophages represent, therefore, ex-vivo model system for the study of immunological processes.

2.4 Molecular characterisation of NRF-KO cells

RNA analysis

Analysis of DNA from tail biopsies showed that KO mice contain a truncated form of the NRF gene in their genome. Transcription from the NRF-KO allele can still be initiated because of the presence of the 5' region and the 1st exon. But since the 2nd exon is deleted together with part of the intron, which contains the splice acceptor, the NRF-KO RNA cannot be processed by splicing. Thus, the KO mRNA contains one intron sequence that is normally removed by splicing. Multiple STOP codons present in the intron region prevent translation of NRF-KO mRNA to a protein of more that 38 amino acids. Transcription from the NRF-KO allele should result in mRNA with the same size as the WT form: 3,9 kb. The NRF flanked mRNA contains in addition the gene for neomycin resistance and is therefore larger in size: 5,2 kb (Figure 2.16). It was not clear wether the artificial NRF-KO RNA is stable enough to be detected. Thus NRF gene expression in primary murine fibroblasts was tested by RNA and protein analysis.

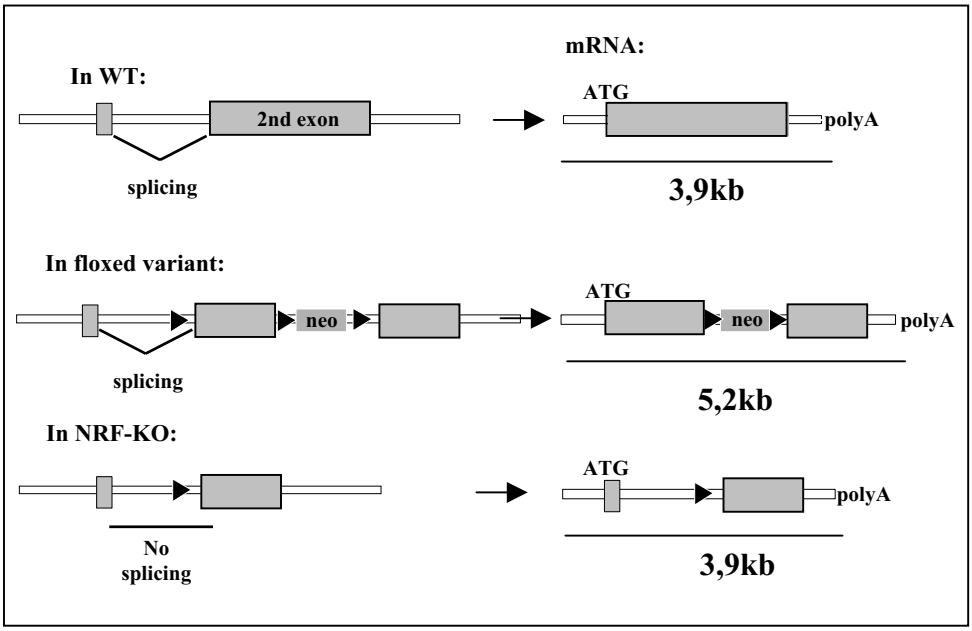

Figure 2.16. *Schematic structure of WT, NRF-floxed and NRF-KO forms of mRNA.*

RNA was isolateded from cultured cells and analysed by Northern blot analysis. Figure 2.17 shows a representative mRNA blot hybridised with 2nd exon specific DNA fragment. As expected, in KO cells no NRF specific mRNA was obtained because of lack of the major part

of the NRF gene. In heterozygous cells containing both WT and NRF flanked alleles two different mRNA were detected, representing the WT and the NRF flox form.

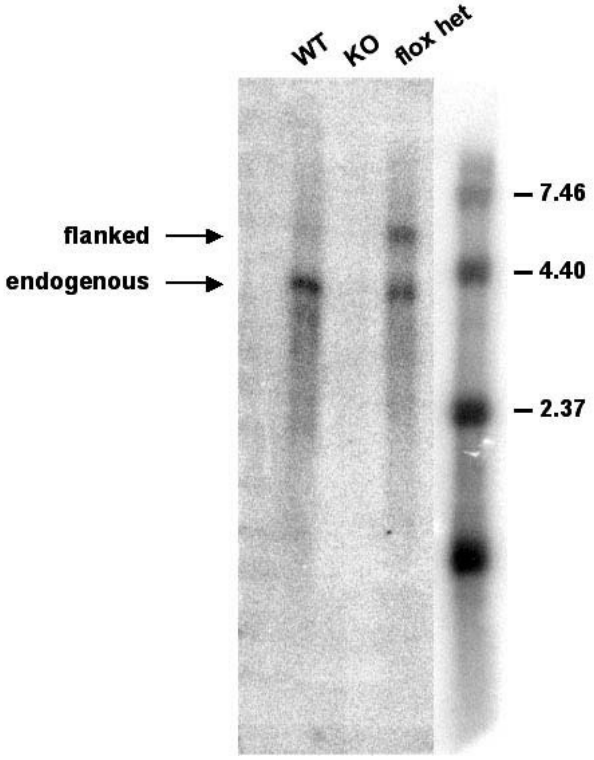

Figure 2.17. *Northern blot analysis. mRNA was isolated from WT, NRF-KO and NRF-flox cells. The RNA blot was hybridized with a radioactively labelled NRF 2nd exon specific probe. For each sample 2mg mRNA was loaded.*

Protein analysis

The NRF protein in WT cells was shown to localize predominantly in nucleoli (Niedick et al, 2004). Therefore, for protein analysis nuclear extracts were isolated from WT and NRF-KO cells and tested by Western Blot analysis using specific antibodies directed against NRF (Figure 2.18, A). The calculated molecular weight of the NRF protein is 77,6 kD. In nuclear lysates isolated from WT cells a specific band just above this size can be detected. The antibody is directed against amino acids 671-690 at the C-terminus of the NRF protein. The coding region of this part is not eliminated in NRF-KO cells and should also be encoded by the mRNA (Figure 2.16). However, it should not become translated into protein because of multiple upstream STOP codons in the intron sequence. Indeed, in lysates isolated from KO cells no protein band was detected confirming the lack of any form of the NRF protein in these cells. To confirm specificity of the antibody the gel was loaded with cell extracts isolated from C243 cells stably overexpressing a myc-tagged-NRF protein. Myc-tagged NRF could be detected by re-incubation of the protein blot with anti-myc antibodies (Figure 2.18, B). The difference in the molecular weight of endogenous NRF and myc-NRF is explained by the myc epitope.

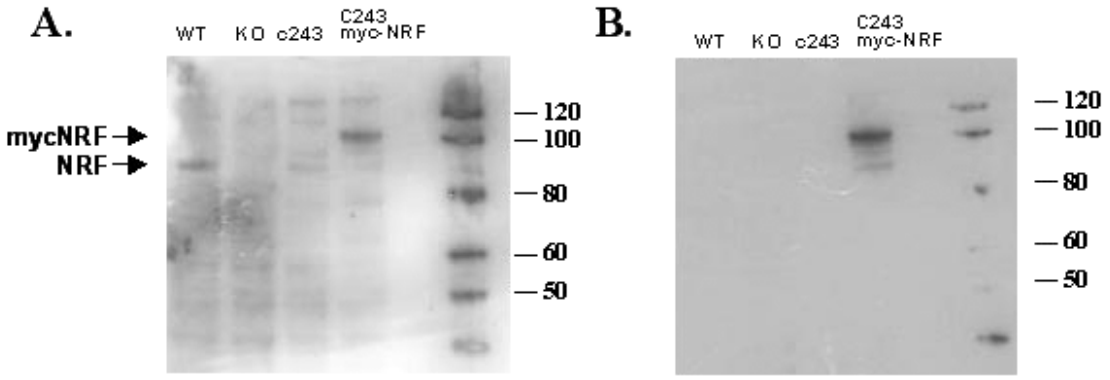

Figure 2.18. *Western blot analysis of NRF expression. Nuclear extracts were prepared from WT and NRF-KO primary cells as well as from a C243 cell line, overexpressing the myc-tagged NRF protein and empty cells. For primary cells 30 mg and for C243 cells 15 mg protein was loaded per lane. The protein blot was incubated with anti-NRF antibodies (A) and in a second round with anti-myc-tag ABs (B).*

Indirect immunofluorescence was used to show the level of protein expression and its localization in fixed cells. WT and NRF-KO cells were fixed on the glass slides and stained with the antibodies directed against the NRF protein (Figure 2.19). In a previous study (Niedick et al, 2004) it was shown that the overexpressed myc-tagged NRF protein is predominantly localized in the nucleolus. The experiment shown in Figure 2.19 demonstrates that the localization of the endogenous NRF protein is also restricted to the nucleolus. In KO cells any specific staining with anti-NRF antibodies was detected, confirming the complete deletion of the NRF protein in these cells. Myc-tag NRF expressing cells were used as a positive control in this experiment (data not shown).

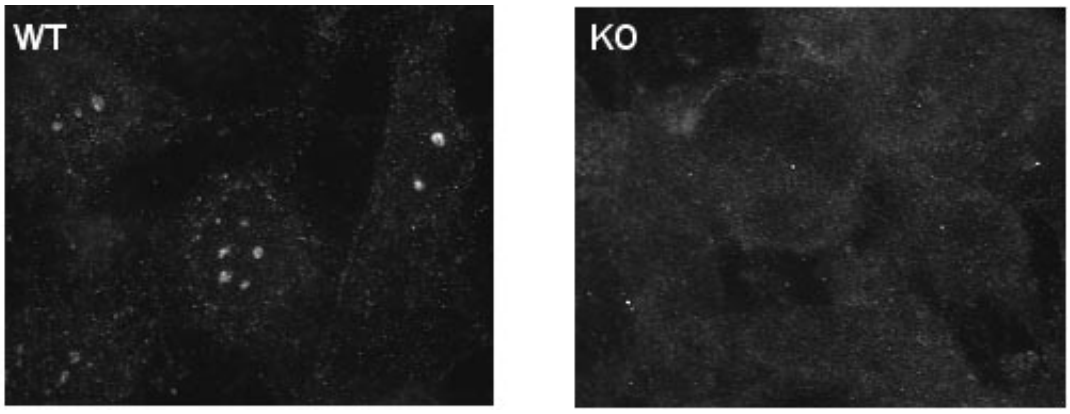

Figure 2.19. *Immunofluorescence. The WT or NRF-KO cells were fixed and then incubated with ABs directed against NRF. The secondary AB was fluorescence labelled. Vizualization was performed by confocal laser scanning microscopy.*

In *in vitro* studies it was shown that the NRF protein specifically binds an NRE sequence in NF-κB regulated promoters. NRE-related sequences have been identified in several promoters, including IFN-β, iNOS, HIV-1 and IL-8. But binding of NRF to the NRE in the IFN-β promoter was best characterized. Human and murine IFN-β promoters are highly homologous. Because of this reason the NRE sequence from the murine (identical to the human) IFN-β promoter was used to study the DNA-binding property of endogenous NRF protein in murine cells. Nuclear extracts isolated from WT, NRF-flox and NRF-KO cells were incubated with the radioactively labelled NRE fragment (Figure 2.20, A). The electromobility shift assay (EMSA) revealed, however, that NRF is not the only factor that binds to the NRE. Protein binding to the NRE was obtained also with lysates from KO cells. Using the unlabelled NF-κB specific sequence as a cold competitor it is shown that NF-κB is the protein that binds to the NRE *in vitro*. Different approaches were undertaken to show specific NRF binding. Unfortunately it could not be clarified whether both proteins bind DNA simultaneously or if they compete for the binding.

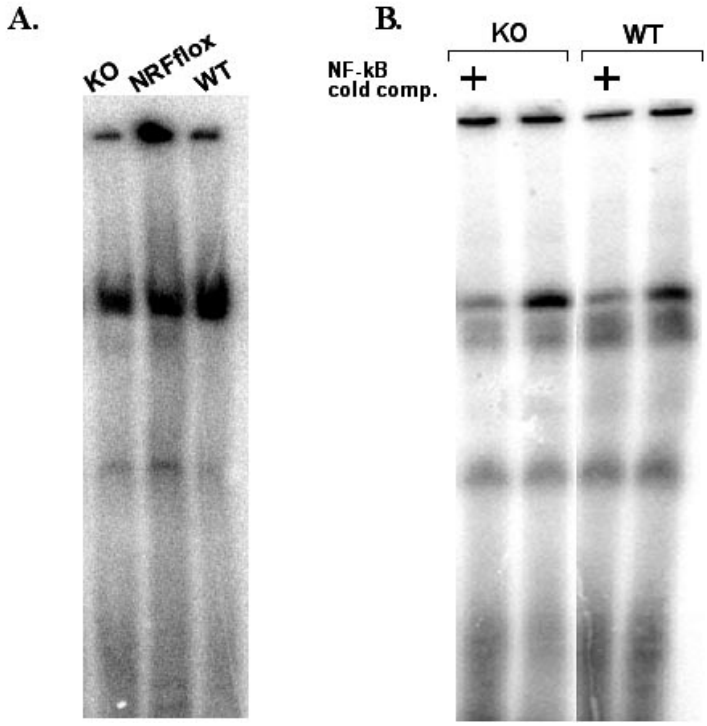

Figure 2.20. *Electromobility shift assay. Nuclear extracts isolated from primary WT, NRF-flox and NRF-KO cells were incubated with radiolabeled IFN-β promoter NRE oligonucleotide (A). Competitions with unlabelled (Cold comp.) NF-κB consensus sequence is also indicated (B).*

Gene expression profile of the NRF-KO cells

The recently released AFFYMETRIX GENECHIP Microarray Technology allows simultaneous and quantitative analysis of the mRNA expression profile for 22629 probe sets, corresponding to more than 15000 genes within a single experiment. For application of this method total cell RNA is fluorescence labelled and used for hybridization on the chip. Binding of labelled mRNA to the appropriate DNA fragment results in a specific fluorescent signal that can be instrumentally quantified. Comparison of the intensity of fluorescent signals from the sample to a control allows the identification and the quantification of differentially regulated genes. The microarray experiment involved the following steps: preparation of fluorescently labelled probes from RNA isolated from WT and KO primary cultured fibroblasts that was labelled and hybridized to the microchips. Afterwards, the chips were scanned and the received images were analysed. From these results the gene expression profiles were deduced. Figure 2.21 shows the expression pattern of KO cells compared to that of WT cells. 25 genes were found to be down- and 17 - up- regulated in NRF-KO cells. However, the degree of this diference is rather low and cannot be regarded as statistically significant. It was therefore concluded that there is no difference between the gene expression pattern in primary cultured WT and NRF-KO fibroblasts. A difference in the expression level of sex chromosome specific genes (inactive X specific transcript, eukaryotic translation initiation factor 2 and DEAD box polypeptide) serves as internal control of the method. WT cells were isolated from the female mice whereas NRF-KO cells are derived from the male animals.

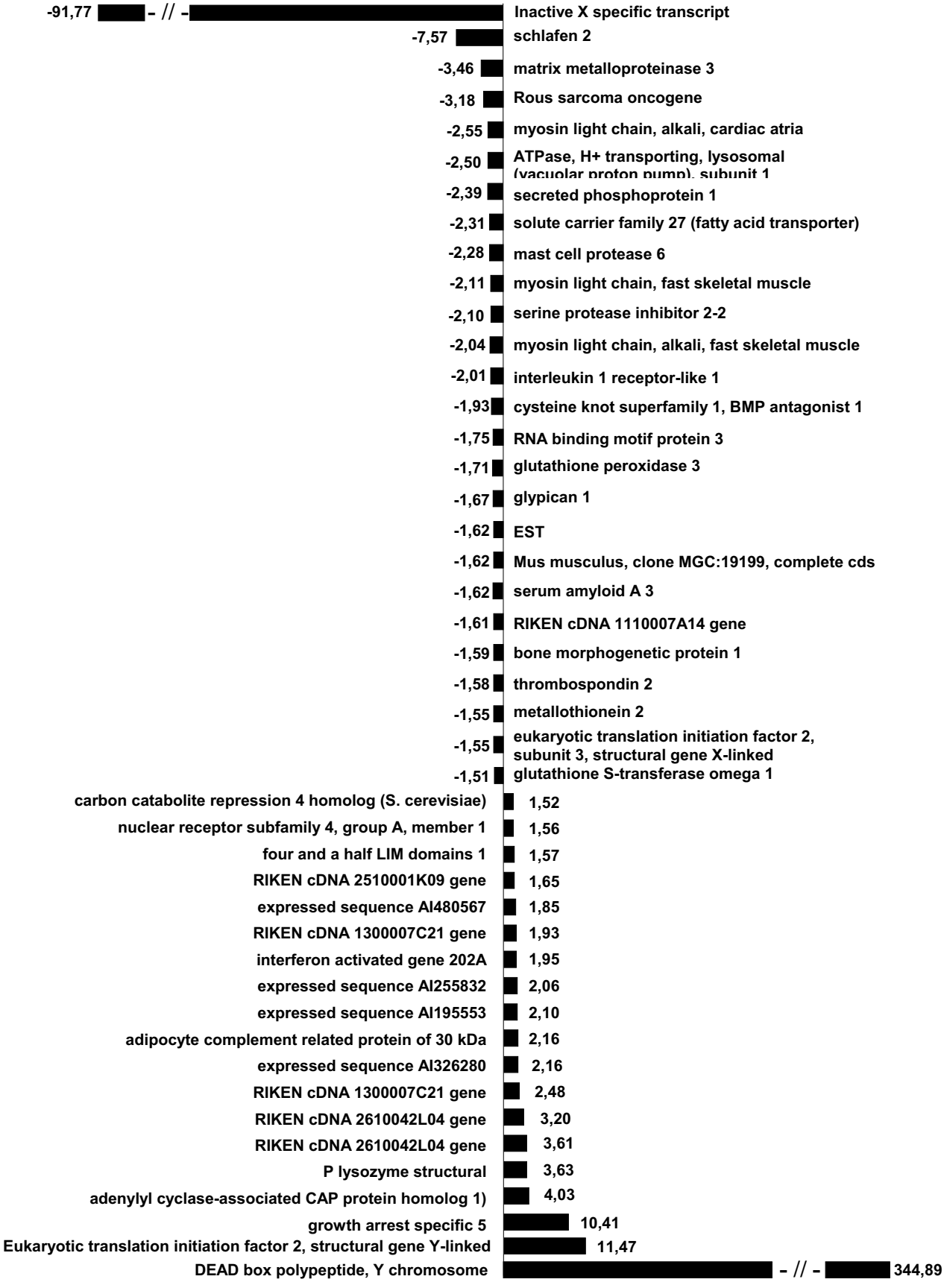

Figure 2.21. *Comparison of gene expression profile of NRF-KO relative to that of the WT in primary cultered fibroblasts. Negative values represent genes, which expression was down-regulated and positive values those with up-regulation in NRF-KO samples.*

Effects of NRF deletion on IFN-β gene expression

NRF was identified as inhibitory factor, binding to the IFN-β promoter and preventing its production in the unstimulated state. Therefore, lack of the NRF protein could lead to some constitutive level of IFN-β production. ES cell were characterised as cells that are not able to produce interferons (Francis et al, 1989). NRF-KO ES cells retain this property. In the supernatants of WT and NRF-KO ES cells stimulated with virus and of those remained unstimulated IFN-β was not detected (data not shown). This effect is explained by a general unability of ES cells to produce IFNs, which is due to an inhibiting mechanism on the epigenetic level in ES cells. However, IFN-β can be produced by nearly all differentiated cell types. Thus, WT and NRF-KO primary fibroblasts were subjected to an IFN antiviral assay. In this assay cells are infected with virus (Newcastle Disease Virus, NDV) for the induction of IFN expression. Then the cells produce IFN for 24 hours and finally supernatants from induced and control uninduced cells are tested for their ability to protect IFN-responsive $LMTK^-$ cells from infection with Vesicular Stomatitis Virus (VSV). $LMTK^-$ cells that do not undergo lysis upon infection with VSV are therefore incubated with supernatants containing sufficient IFN to protect the cells. Comparison of the ability to protect $LMTK^-$ cells with IFN from the supernatants with that of a standard IFN-β sample, the concentration of IFN produced by the test cells can be determined. In Figure 2.22 crystal violet staining of the $LMTK^-$ cells 24h after infection with VSV is shown. As it can be seen, cells that were incubated with supernatants from control, non-induced WT, NRF flox and KO cells did not survive the VSV infection. This indicates that NRF-KO cells do not constitutively produce IFN-β or the level of this production is undetectable with this method. On the other hand the IFN-β production by the cells stimulated with NDV is similar in WT and NRF-KO cells.

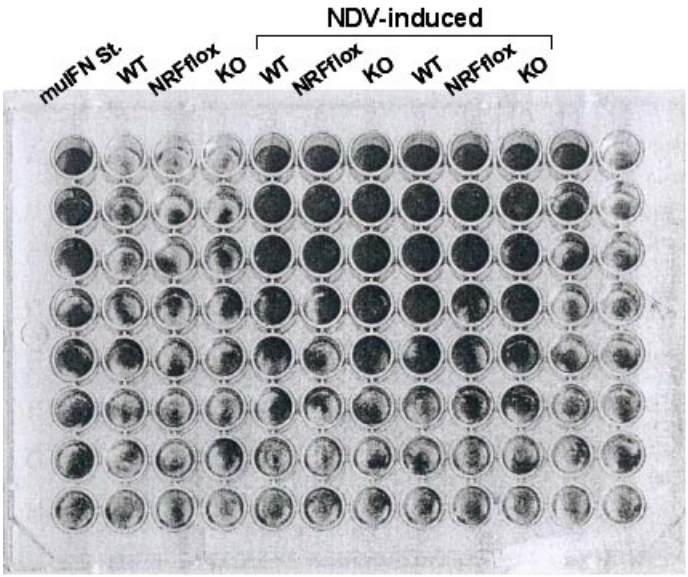

Figure 2.22. *Test for IFN production. LMTK cells were incubated for 24h with supernatants from WT, NRF flox and NRF-KO cells that were pre-induced with NDV or left untreated (as indicated). LMTK⁻ cells were subjected VSV infection and 24h later stained with crystal violet. Cells in the first line were incubated with standard murine IFN-β.*

Regulation of IFN-β is strictly controlled and even low levels of a basal expression might have physiological effects. A more sensitive method than the antiviral test is an analysis of gene expression with RT-PCR. Total RNA isolated from WT and NRF-KO cells was reverse-transcribed and then tested by PCR using IFN-β specific primers (Figure 2.23). With this method no IFN-β production by NRF-KO cells was found. We also tested with this method the endothelial cells we obtained by in vitro differentiation of ES cells (see chapter 2.3), but with the same result. As a positive control RNA isolated from mouse embryonic fibroblasts (MEFs) was used. These cells are known for their ability to produce low level of IFN-β in the unstimulated state (Zawatzky, not published).

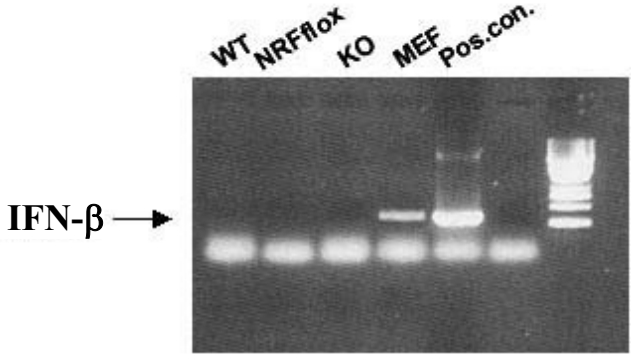

Figure 2.23. *RT-PCR. RNA was isolated from cultured WT, NRF flox, NRF-KO and MEFs. mRNA was reverse-transcribed into cDNA and subjected to PCR analysis using IFN-β specific primers. Pos. con.=positive control that represents genomic DNA.*

Effects of NRF deletion on iNOS activity

Another gene, whose expression is regulated by NF-κB and that was shown to have an NRF binding site in its promoter is inducible Nitric Oxide Synthase (iNOS) (Feng et al, 2002). The iNOS gene is transcriptionally silent in the unstimulated state, but can been rapidly induced

by different agents, for example, by LPS. As a result of iNOS activity Nitric Oxide (NO) is produced. This molecule plays an important role in antibacterial and antiviral immunity. Because of the proposed modulatory role of NRF for transcriptional repression of iNOS gene it was hypothesized that a lack of the NRF inhibitory action in the iNOS promoter could cause some expression level of this enzyme in unstimulated conditions and lead to accumulation of NO. The cells known for their ability to express high level of iNOS are endothelial cells and macrophages. Native peritoneal macrophages from WT and NRF-KO mice were isolated and analysed for possible constitutive iNOS activity. The sensitive RT-PCR analysis was performed using iNOS specific primers. However, no basal expression of the mRNA could be detected (data not shown). To check the induction of iNOS activity in freshly isolated NRF-KO primary macrophages these cells were induced with 10 μg/ml of LPS and analysed for NO production. NO is an unstable molecule with a very short half-life time and can therefore not be detected directly. By oxygen and water NO is rapidly converted into the more stable NO_2 molecule. The NO metabolite NO_2 accumulates in the extracellular space and can be quantitatively measured. Its concentration correlates with level of NO production by the test cells. Figure 2.24 shows the rate of measured NO_2 in WT and NRF-KO cells 24 and 72 hours after induction with LPS.

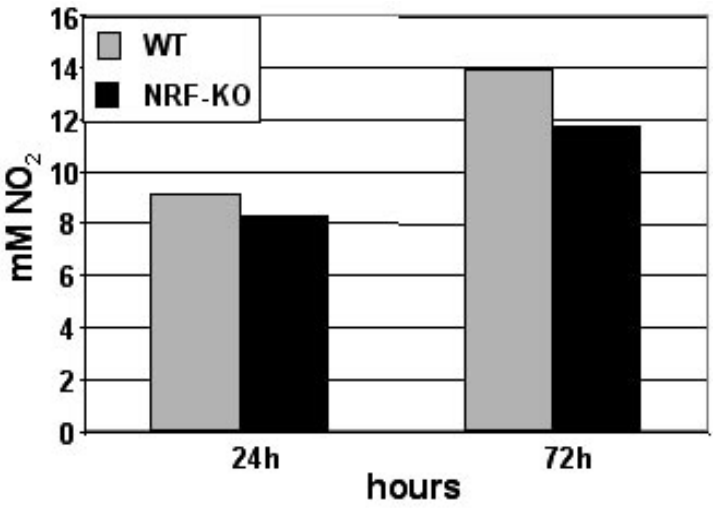

Figure 2.24. *NO_2 production by macrophages. Freshly isolated WT (grey bars) and NRF-KO (black bars) macrophages were stimulated with 10mg/ml LPS. NO_2 was measured in the supernatants 24 and 72 hours after stimulation with Griess reagent.*

This experiment indicates that iNOS expression can be induced in cultured primary macrophages. NO production can be measured already 24h after stimulation, but is

significantly increased 72 h after stimulation and the induction level is similar in WT and NRF-KO cells.

In experiments described above a possible effect of the lack of the NRF protein was tested with the model of in vitro cultured NRF-KO cells. In contrast to the expectation, we couldn't detect any basal expression of IFN-β or iNOS genes in unstimulated NRF-KO cells. NRF-KO cells were isolated from the normally developed newborn (fibroblasts) or adult (macrophages) mice. Both, IFN-β and iNOS, are strictly regulated genes with a number of factors involved in their control. We hypothesize, that during embryogenesis mechanisms might be activated to compensate the lack of the NRF protein. Such mechanisms could involve over-activity of other factors participating in the constitutive silencing of NF-κB regulated promoters. So that compensatory mechanisms override the immediate phenotype of the eliminated NRF gene. This can be tested *in vitro.* To do so, we decided to delete the NRF gene in cultured cells using the Cre-loxP system. For this purpose in primary cells which are homozygous for the NRF floxed allele the Cre recombinase must be expressed. For this a method that allows efficient and rapid excision of a functional floxed locus was needed. Because primary cells are usually difficult to transduce our method of choice was retroviral infection and subsequent selection for the successfully treated cells.

2.5 *In vitro* excision of NRF gene in primary cells by retroviral Cre transduction

Retroviruses

Retroviral infection is a DNA transfer technique that allows efficient introduction of nucleic acids in diverse cell types, even where the general transfer methods like transfection and electroporation fail.

A specific feature that characterizes all retroviruses is that a single particle contains two copies of the single stranded genomic RNA, which after infection of the target cell is reverse transcribed to the complementary double stranded proviral DNA that integrates into the host genome. Proviral DNA terminates at the both 5' and 3'ends by two identical long terminal repeats (LTRs). This region contains the control promoter/enhancer elements for viral gene expression. The viral genome contains genes that essential for viral replication, usually there are 3 genes, encoding for precursor of capsid protein (*gag* and *env*) and protease responsible for the cleavage of gag polyproteins (*pro*). Retroviral vectors are constructed in a way that the

structural viral genes are replaced by the gene of interest. Upon integration into the genome of infected cells introduced nucleic acids are transcribed, but new viruses will not be assembled because of the lack of essential proteins. For the production of the retroviruses packaging cells were developed, which provide the retroviral proteins by expression in *trans*.

Construction of the retroviral vector

A retroviral vector for the constitutive expression of a GFP-Cre fusion protein was constructed (Figure 2.25). The fusion of the Cre recombinase to GFP allows analysis of infected cells by FACS and sorting effectively transfected cells. Expression of the GFP-Cre fusion protein is driven by the retroviral internal LTR promoter. The GFP-Cre gene is followed by the gene encoding the neomycin resistance under the translational control of IRES (internal ribosomal entry site), that allows additional selection.

Figure 2.25. *Schematic presentation of retroviral construct encoding for GFP-Cre fusion protein.*

Establishment of the producer cell line

The packaging cell line GP+E86 was transfected with the GFP-Cre retroviral vector. To get stable virus producers the transfected cells were selected for neomycin resistance. A titer of pM5GFPCreneo transfected packaging cells was determined on mouse fibroblasts NIH3T3 cells. The titer was $2{,}8 \times 10^5$ cfu/10^6 cells in 24 hours.

Expression analysis

The efficiency of infection with pM5GFPCreneo was tested on the mouse fibroblast cell line NIH3T3, to find optimal conditions for infection. Infection with different MOI (multiplicity of infection) was tested. The MOI indicates virus/cell ratio. The best infection efficiency was reached when 2×10^4 cells were infected with 10^5 viruses (MOI = 5).
The kinetics of infection with pM5GFPCreneo was also tested. GFP expression was measured in the infected cells 24, 48 and 72 hours after infection. The level of GFP expression was analysed by FACS (Figure 2.26).

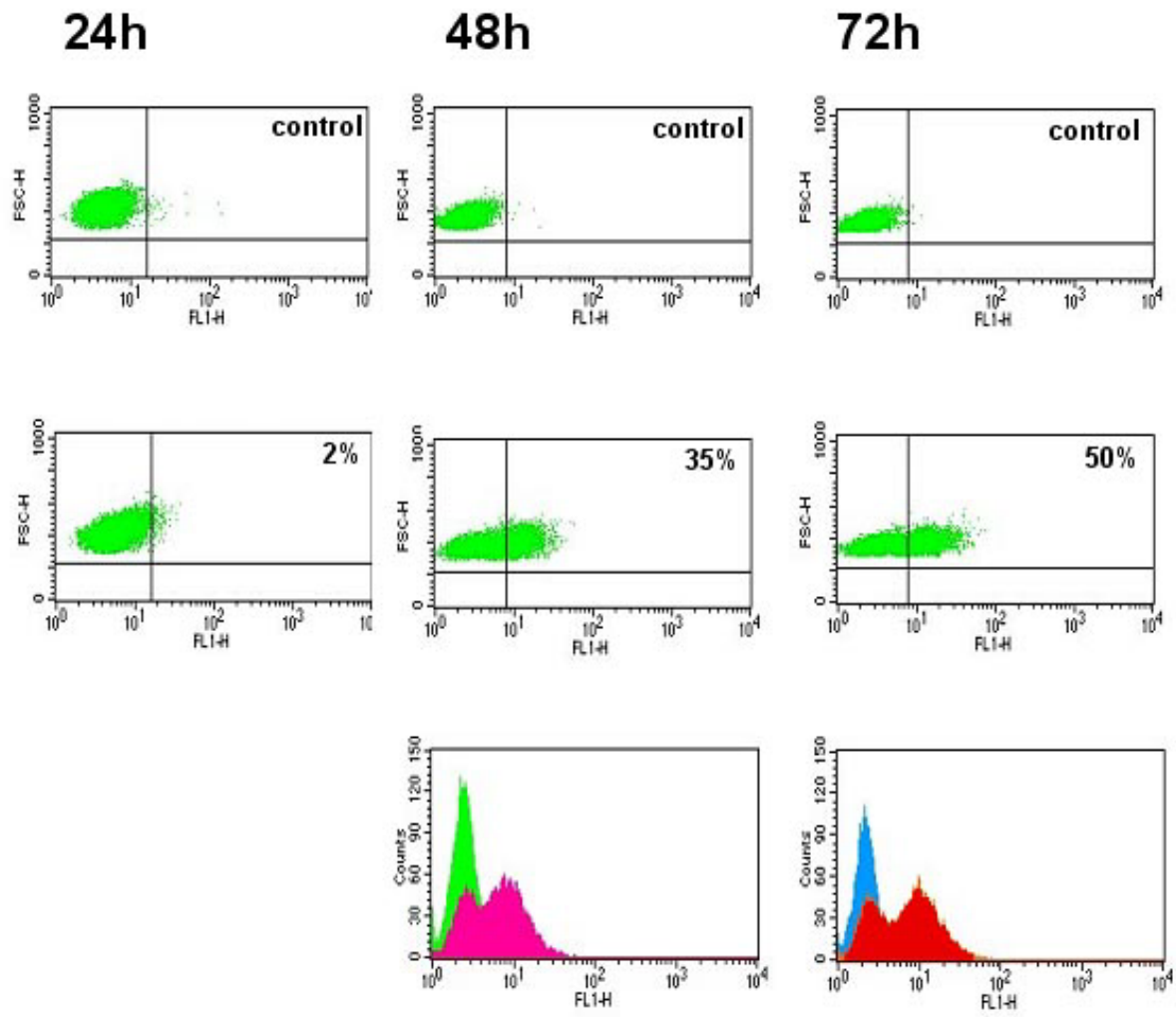

Figure 2.26. *Kinetics of GFP expression after infection. NIH3T3 cells were infected with supernatant containing pM5GFPCreneo virus with MOI=5. 24, 48 and 72 hours after infection GFP expression by the cells was measured by FACS.*

At 24 hours after infection expression of GFP-Cre protein was not detectable. 48h after infection a significant level of GFP-Cre expression (35%) was reached. This level was increased 72h after infection (50%).

Infection of primary cells

The freshly isolated mouse skin fibroblasts (see chapter 2.3) from WT and NRF-flox mice were infected with pM5GFPCreneo retrovirus at the same conditions that were established for NIH3T3 cells. 24, 48 and 72 hours after infection the cells were tested for GFP expression (Figure 2.27, A).

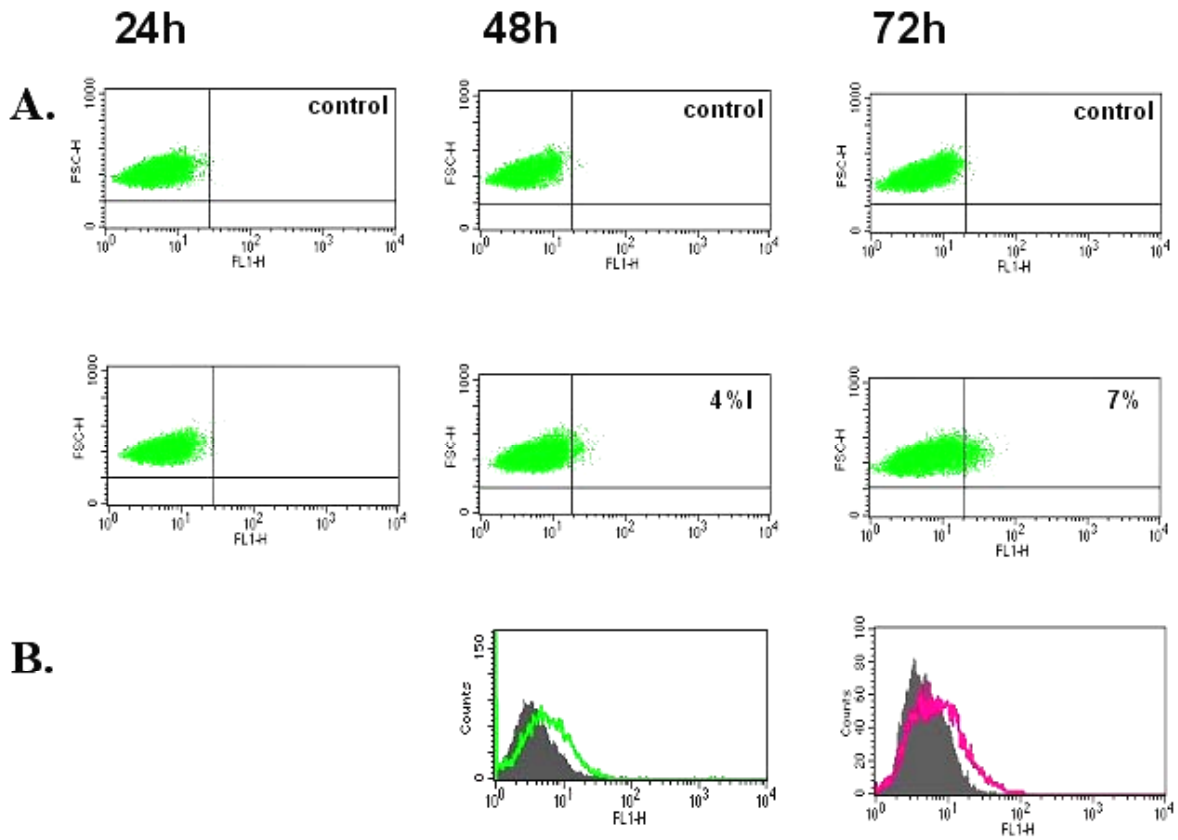

Figure 2.27. *GFP-Cre expression in primary fibroblasts. Cells isolated from the skin of newborn mice were infected with supernatant containing pM5GFPCreneo virus. In indicated time after infection (24, 48 and 72 hours) infected cells were measured by FACS for GFP expression (A). B. Overlaying histograms of control (grey area) and infected cells 48h after infection (green line) and 72h after infection (pink line).*

The level of the GFP expression was significantly lower as that shown for NIH3T3 cells: about 4% 48h after infection and 7% 72h after infection. However, the overlaying histogram (Figure 2.27, B) shows that most of infected cells are slightly shifted towards GFP positive cells. This indicates that most cells exhibit some, but a relatively low level of GFP expression. Thus, the low percentage of GFP positive cells is caused by technical difficulties to detect GFP expression in primary cells. Another reason is that the used GFP fused to Cre is the WT-GFP and not an enhanced form of the protein (eGFP).

Based on the data from FACS analysis (Figure 2.27) one could imagine that upon infection with pM5GFPCreneo retroviruse even cells that are not detected as GFP positive express some GFP-Cre fusion protein. This could subsequently lead to Cre mediated excision of the NRF gene. To check the ability of GFP-Cre fusion protein to delete the flanked gene infected

cells were analysed with PCR. DNA was isolated from NRF flox cells 72 hours after infection and analysed with NRF flox and KO specific primers (Figure 2.28).

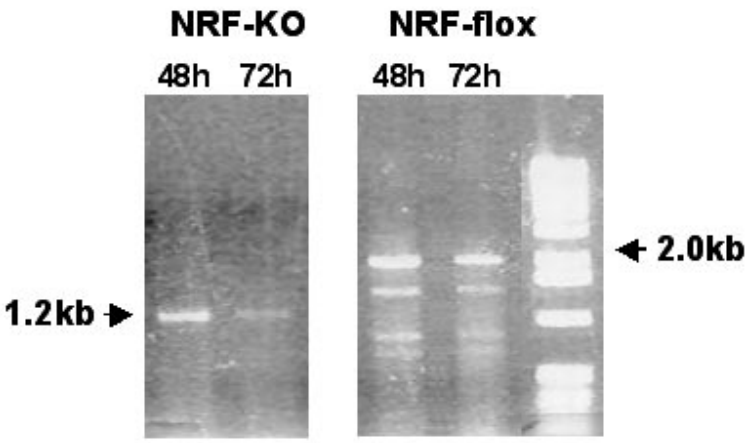

***Figure 2.28**. PCR analysis of GFP-Cre virus infected NRF flox cells. DNA was isolated from NRF flox cells 48 and 72 hours after infection and tested with NRF flox and KO specific primers.*

An 1,2 kb band is specific for the NRF deletion. The presence of this band indicates that Cre recombinase fused to GFP protein is functionally active and mediates excision of the flanked cassette. However, there is still a population of cells with insufficient level of Cre expression and these cells retained the NRF flox genotype (indicated by the 2,0 kb band).

Thus, retroviral infection of primary fibroblasts with GFP-Cre viruses results in a mixed population of uninfected or insufficiently infected NRF flox cells and those that have successfully undergone Cre mediated excision. In order to get a homogenous population of cells to study the NRF gene deletion it is essential to separate NRF-KO from the rest of the cells. The fusion of the Cre recombinase to GFP allows the sorting of the infected cells by Flow cytometry and to obtain a homogenous populations of Cre expressing cells. An experiment was performed in which WT and NRF flox cells were infected with pM5GFPCreneo virus followed by sorting for GFP expression 72 hours after infection. A respresentative experiment is shown in Figure 2.29. 12% GFP positive WT cells and 25% GFP positive NRF flox cells were obtained. In all experiments the value of GFP positive cells fluctuates between 10 and 30% and does not depend on the cell type (WT or NRF flox). The sorted cells were cultured further 6 days and RNA was isolated.

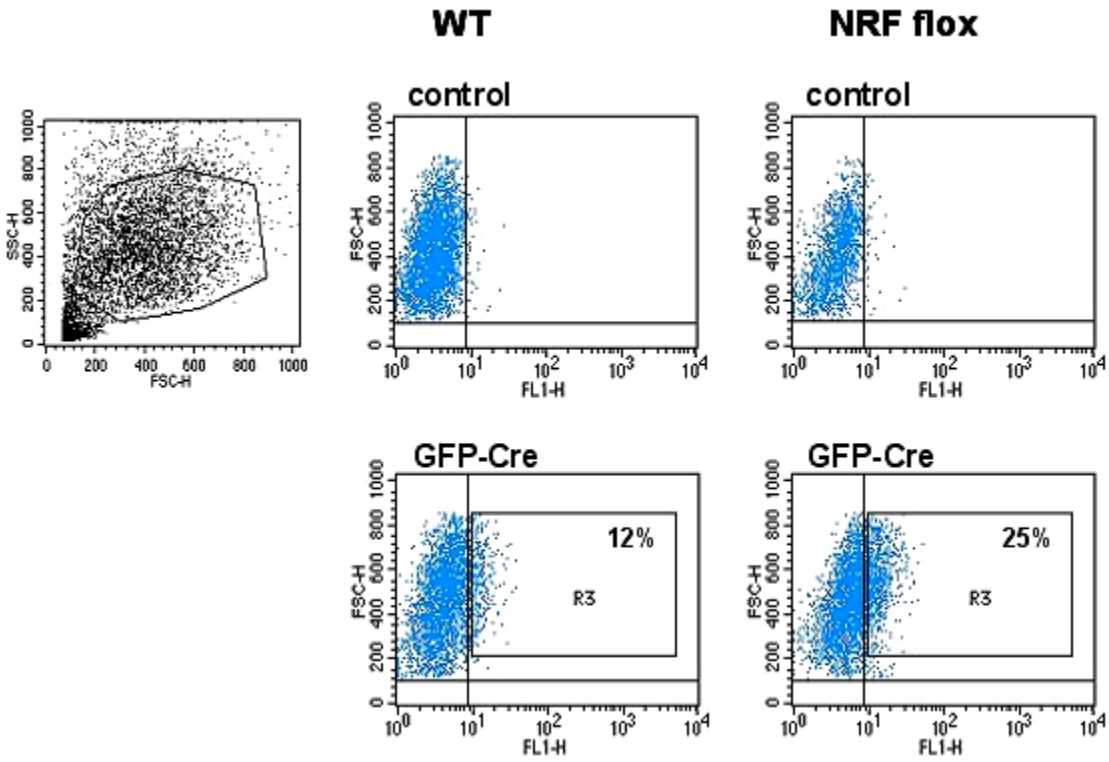

Figure 2.29. *Sorting of GFP-Cre infected cells. WT and NRF-flox primary fibroblasts were infected with GFP-Cre virus. 72 hours later GFP positive cells were isolated by cell sorting.*

The deletion of the NRF gene was tested by RT-PCR. For detection of NRF-KO cDNA with PCR one of the primers was intron specific, the other was located on the 3'-end (Figure 2.30). In parallel PCR for detection of WT and NRF flox alleles was performed.

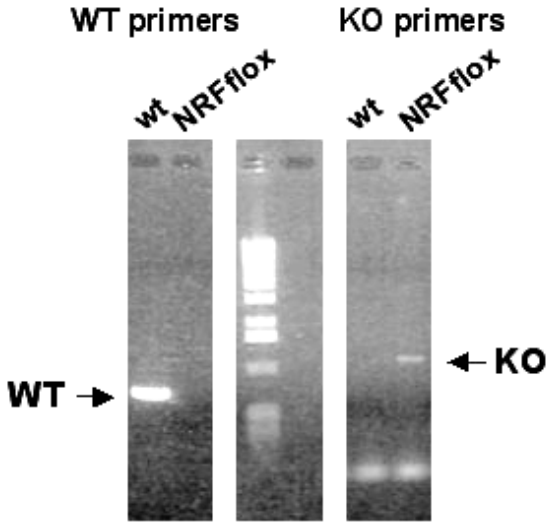

Figure 2.30. *Characterization of cells with the in vitro deleted NRF gene by RT-PCR. RNA was isolated from sorted GFP positive cells (WT and NRF flox), produced by infection with GFP-Cre virus. mRNA was reverse-transcribed into cDNA and subjected to PCR analysis using NRF-WT and NRF-KO specific primers.*

As expected, in WT cells GFP-Cre infection does not affect NRF mRNA expression and only the WT mRNA is found. In the sorted NRF flox cells no sign of RNA from the floxed allele is detected, but only the product corresponding to the NRF excision. Thus sorting of GFP

positive cells upon infection with GFP-Cre retroviruses results in a pure population of cells that express the GFP-Cre at levels sufficient for complete Cre-mediated excision of the flanked allele.

The homogenous population of NRF-KO cells is produced from cells that carried the NRF flanked allele (by retroviral Cre transduction) in cultured primary cells within a few days. To investigate whether this sudden excision of the NRF gene causes immediate activation of NF-κB regulated promoters the expression level of IFN-β in these cells was analysed. RNA isolated from WT and NRF-KO cells that were sorted upon infection with GFP-Cre expressing retroviruses was examined by RT-PCR using IFN-β specific primers (Figure 2.31). Neither WT nor the freshly deleted NRF-KO cells express IFN-β in the non-stimulated state. As a positive control for RT-PCR the expression of the housekeeping gene GAPDH was confirmed (Figure 2.31).

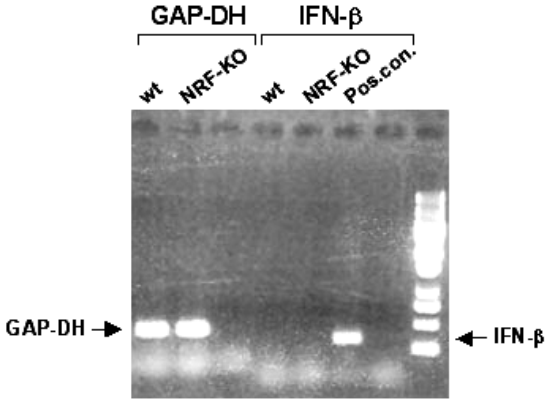

Figure 2.31. *IFN-β expression assay of freshly deleted NRF gene in primary cells by RT-PCR. RNA was isolated from sorted GFP positive cells (WT and NRF-KO), produced by infection with GFP-Cre virus. mRNA was reverstranscribed into cDNA and subjected to PCR analysis using IFN-β and GAPDH specific primers. Pos. con.=positive control and represents genomic DNA.*

This result let us conclude that even in freshly deleted NRF-KO cells the lack of NRF protein alone is not sufficient for activation of promoters, in which NRF is supposed to have an inhibitory role. Thus, the sudden deletion and deletion in the early embryonal development do not differ in this respect.

2.6 Overexpression of NRF in primary cells

To study effects of excessive expression of the NRF the protein was overexpressed in primary cells. For this purpose a retroviral vector for the constitutive expression of NRF was constructed (Figure 2.33).

Figure 2.33. *Schematic presentation of retroviral construct encoding for myc-tagged NRF protein.*

For detection of the NRF protein a myc-tag was coupled to the N-terminus of the NRF protein. The myc-epitope represents six repeats of 13 amino acids, each representing a recognition site for the available antibody. This epitope is derived from the c-myc protein and can be specifically detected with anti-myc-tag antibodies. Expression of the mRNA is driven by the retroviral LTR promoter. The NRF reading frame is followed by the reading frame encoding a fusion protein that is composed of GFP, thymidine kinase and gene for neomycin resistance (GTN). Its translation is under the control of an IRES (internal ribosomal entry site) element. This fusion protein allows multiple selection procedures.
The virus producing cell line was established by stable transfection of the packaging cell line GP+E86 with pM5mycNRFgtn retroviral vector.

The efficiency of pM5mycNRFgtn infection was tested on primary cultures of mouse fibroblasts. The cells were infected with pM5mycNRFgtn virus and 48 hours later GFP expression was analysed by FACS (Figure 2.34). Nearly 50% of all infected cells were defined as GFP positive, indicating a high rate of infection.

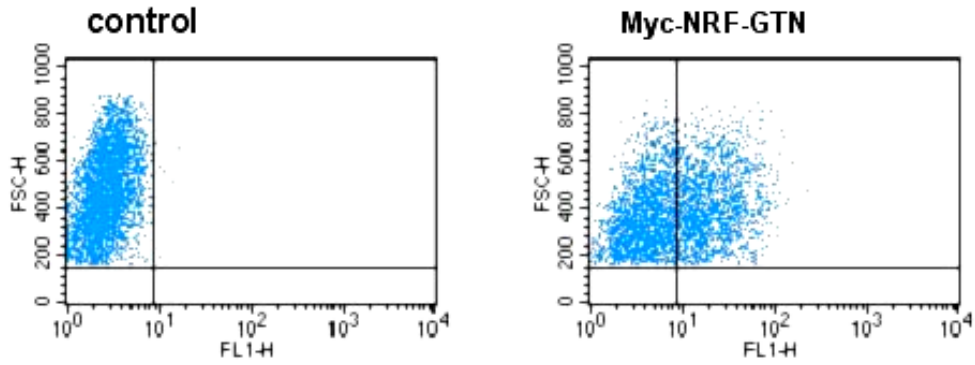

Figure 2.34. *Primary fibroblasts isolated from the skin of newborn mice were infected with supernatant containing pM5mycNRFgtn virus. 48 hours after infection cells were measured by FACS for GFP expression.*

To check the localization pattern of overexpressed NRF introduced by retroviral infection, the pM5mycNRFgtn-infected cells were stained with anti-myc-tag antibodies and analysed by immunofluorescence (Figure 2.35). The localization pattern of retroviraly overexpressed NRF is completely identical with that of endogenous protein which is concentrated in the nucleoli.

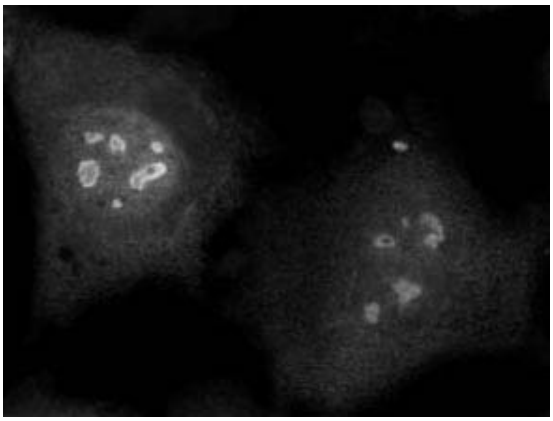

Figure 2.35. *Immunofluorescence. The primary WT fibroblasts were infected with supernatant containing pM5mycNRFgtn virus. 48 hours after infection cells were fixed and GFP expression was analysed by confocal laser scanning microscopy. In both, high expressors and in the cells that express the NRF protein in lower level, the same expression pattern was obtained.*

Thus, the endogenous NRF protein as well the overexpressed one by different gene transfer methods in various cell lines localizes to the nucleolus.

2.7. Effects of NRF deficiency and overexpression on the gene expression pattern

In order to determine the genes whose expression is directly affected by NRF, we applied RNA to Microarray Analysis. Microarray technology allows large scale profiling of gene expression. For this analysis primary mouse fibroblasts isolated from WT, NRF-flox and NRF-KO mice were used as it is outlined on the Figure 2.36.

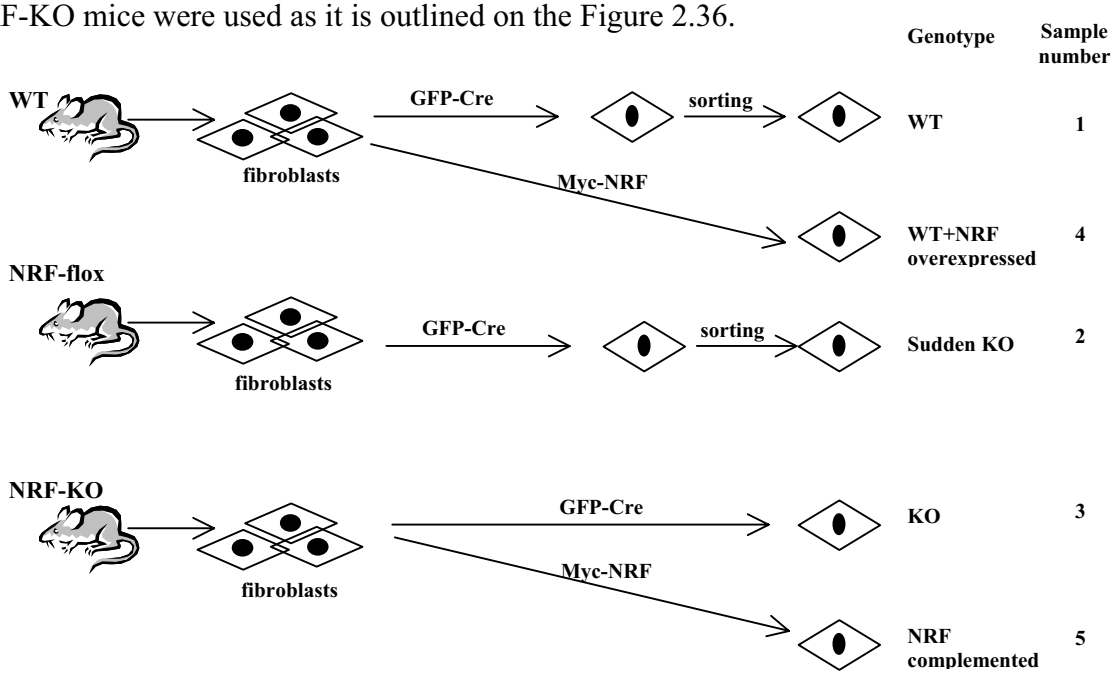

Figure 2.36. *Schematic presentation of the samples used for analysis with Microarray technology.*

To test effects of sudden NRF deletion NRF-flox cells were infected with the GFP-Cre expressing retroviruses with subsequent sorting of GFP positive cells (sample 2). For a control WT cells were used that were subjected to the same experimental procedure (sample 1). To compare effects of NRF gene rapid deletion with a constitutive deficiency NRF-KO cell were infected with the same virus, but not sorted (sample 3). In addition, WT and NRF-KO cells were infected with myc-NRF expressing virus to monitor direct effects of NRF overexpression (samples 4 and 5). Total cell RNA was isolated from the test cells and applied to AFFYMETRIX GENECHIP Microarray Analysis. Image files were analysed using Affimetrix software that included three steps: background adjustment, normalisation and summarisation. Thereafter the data was analysed bioinformatically. Cluster analysis was performed using programme "Genesis". Cluster analysis groups together genes with comparable patterns of expression by employing mathematical methods of similarity organise patterns of expression. Such analysis of NRF deficient or overexpressed samples gives 10 clusters with differently regulated gene expression pattern (Figure 2.37).

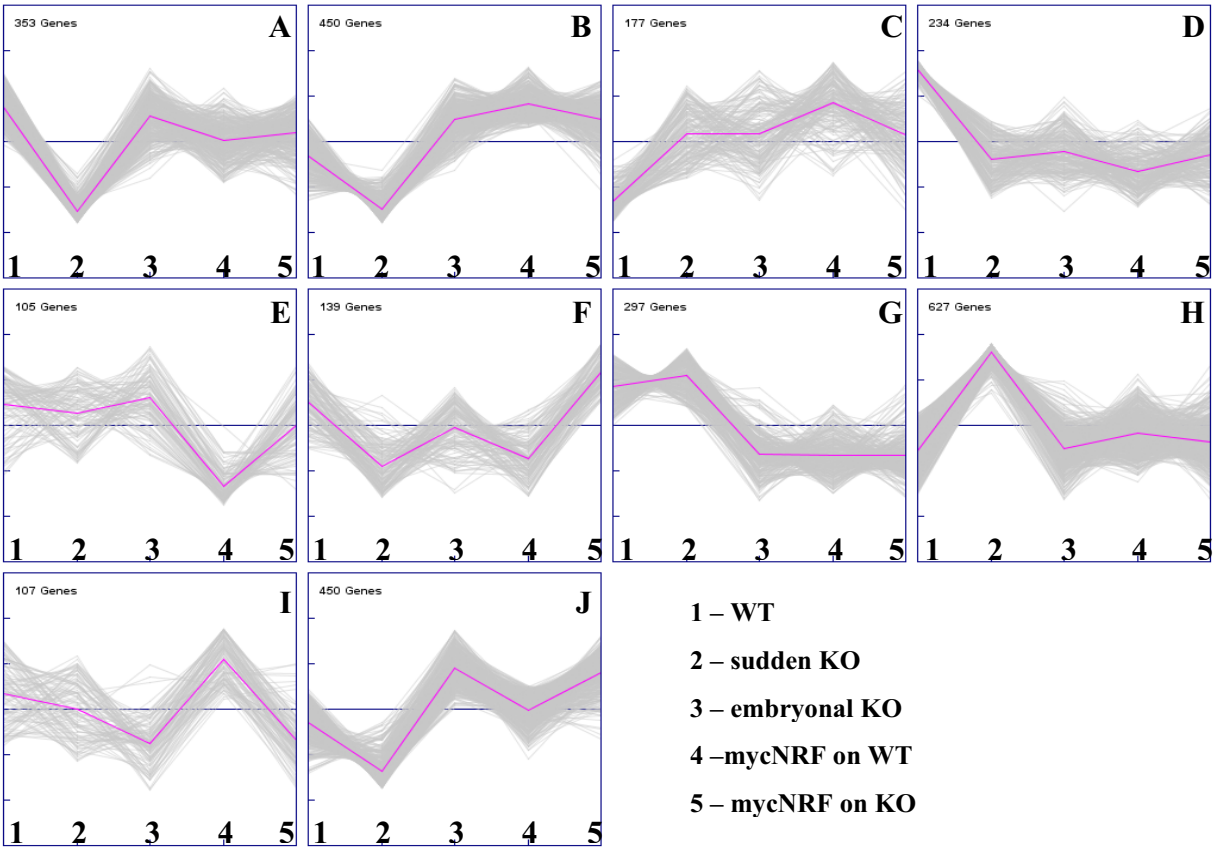

Figure 2.37. *Cluster analysis of genes affected by changes in the NRF expression.*

Clusters A, B and J group genes that are downregulated by sudden inactivation of NRF (sample 2), but remain unaffected in NRF overexpressing cells (samples 4, 5) as well as in

NRF-KO cells (sample 3). Figure 2.38 highlights the genes within this cluster. In contrast, cluster H contains the genes, which expression is up-regulated by sudden deletion of the NRF. Genes regulated by overexpression of the NRF protein are summarized in cluster C (up-regulation) and cluster E (repression), comparing WT cells infected with myc-NRF virus (sample 4) with WT GFP-Cre expressing cells (sample 1). Genes of these expression pattern are shown on Figures 2.39.

To classify clustered genes affected by changes in NRF expression to functional network additional analysis of gene ontology was performed using GeneMap and Go Surfer software. Figure 2.40 shows one example of the affected genes in apoptosis regulation signaling, comparing “sudden” NRF-KO cells with WT cells. Deletion of NRF leads to inhibition of expression of some proapoptotic genes (CytC, caspase3) as well as NF-κB activating kinase IKK. This correlates with upregulation of Mdm2 that acts as a ubiquitin ligase and targets p53 for destruction by proteasomes and promotes p53 degradation (Figure 2.40, A). On the other hand overexpression of the NRF directly up-regulates one of the key proapoptotic genes p53 (Figure 2.40, B). Regarding the influence of NRF deficiency on genes of the immune system we found both up- and downregulation effects. The most interesting data is summarized in tables 2.41 and 2.42.

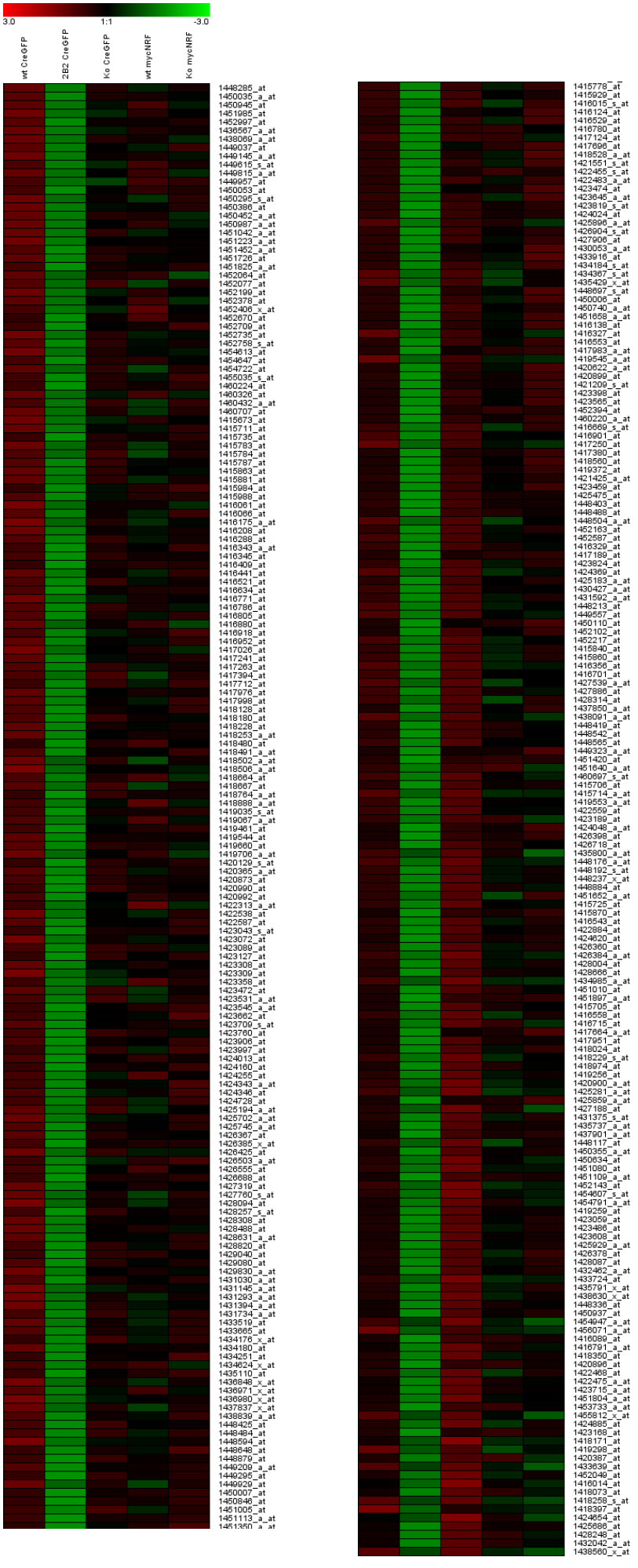

Figure 2.38. *Genes that were organized in cluster A.*

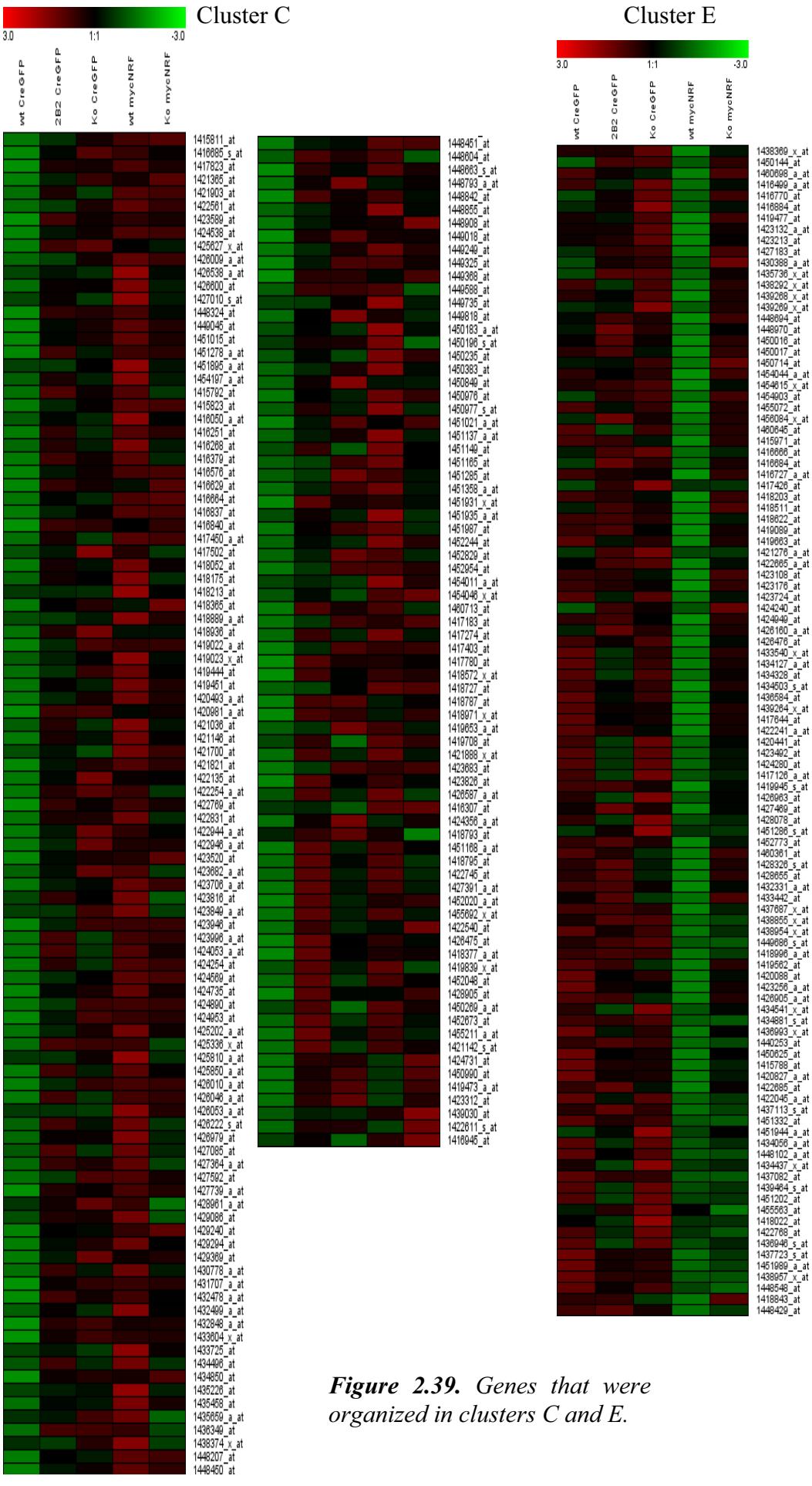

Figure 2.39. *Genes that were organized in clusters C and E.*

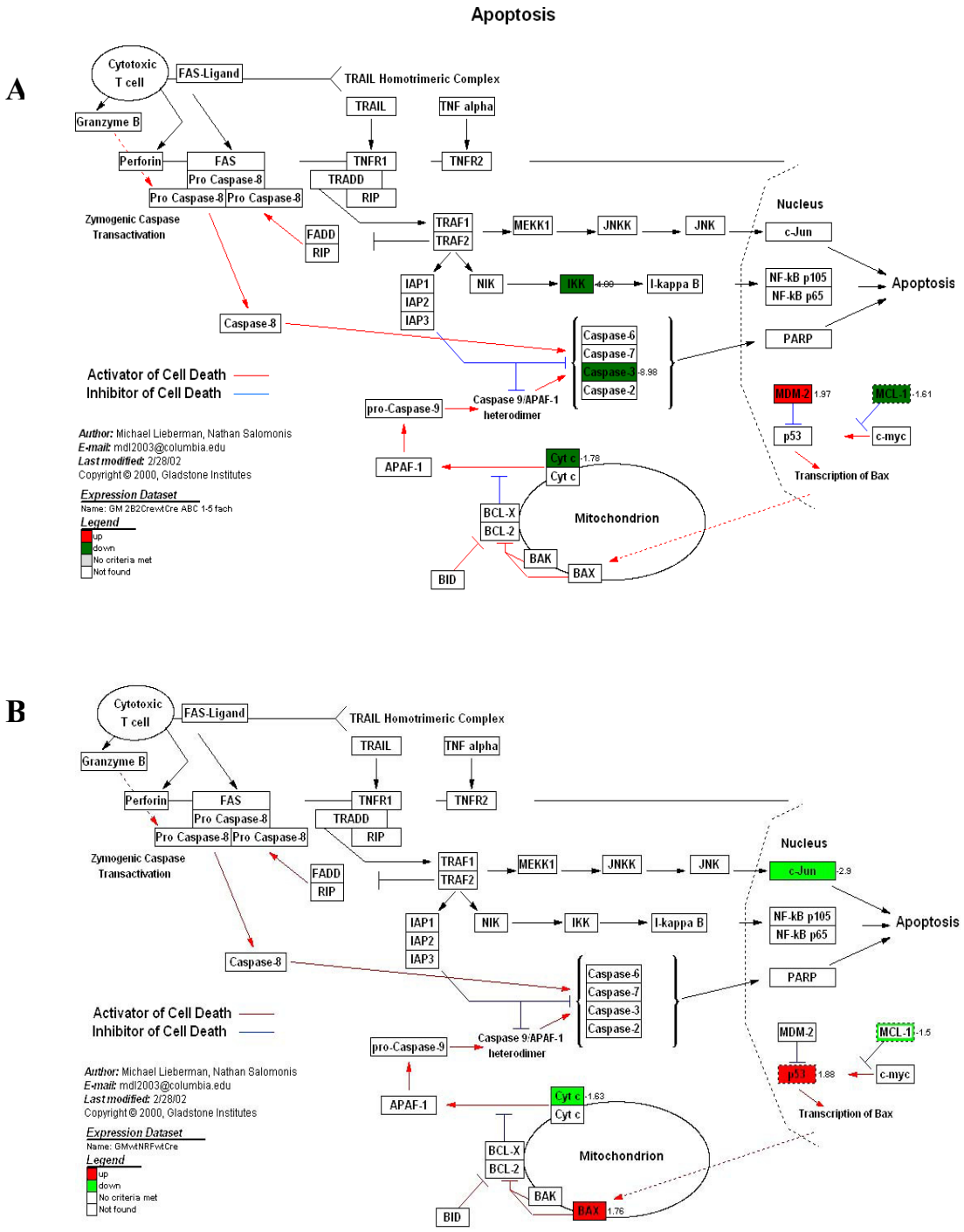

Figure 2.40. *Factors of the apoptosis signaling pathway affected by NRF deletion (A) and NRF overexpression (B). Red marked are genes which expression is up-regulated; green marking represents the down-regulated genes.*

Table 2.41. *Genes of the immune response system undergone up- or down-regulation upon rapid deletion of the NRF.*

Gene symbol	Affy regulation	Unigene title
Fcgrt	2,2	Fc receptor, IgG, alpha chain transporter
Ccl8	3,2	Chemokine (C-C motif) ligand 8
Il20	15,4	Interleukin 20
Mail-pending	2,1	Molecule possessing ankyrin-repeats
Saa3	5,3	Serum amyloid A3
Gadd45g	2,3	Growth arrest and DNA-damage inducible 45
Ada	-2,3	Adenosine deaminase
Ifi202b	-3,3	Interferon activated gene 202B
Swap70	-1,8	SWAP complex protein, 70 kDa
Bcap31	-2,6	B-cell receptor-associated protein 31

Table 2.42. *Genes of the immune response system undergone up- or down-regulation upon NRF overexpression.*

Gene symbol	Affy regulation	Unigene title
Cxcl5	2,1	Chemokine (C-X-C motif) ligand 5
Illr11	1,8	Interleukin 1 receptor-like 1
Cd44	1,6	CD44 antigen
Bcl10	2	B-cell leukemia/lymphoma 10
Gadd45g	1,7	Growth arrest and DNA-damage-inducible 45 gamma
Ly6a	-1,6	Lymphocyte antigen 6 complex, locus A
B2m	-1,7	Beta-2 microglobulin
Ccl8	-1,9	Chemokine (C-C motif) ligand 8
Pla2g7	-2,3	Phospholipase A2, group VII (platelet-activating factor)
Bmi1	-2,6	B lymphoma Mo-MLV insertion region 1

3. Discussion

3.1 Creation and characterization of NRF-KO mice

mRNA of NF-κB repressing factor NRF was shown to be abundant and ubiquitously expressed in all tested cell lines and adult tissues (Nourbakhsh and Hauser, 1999; Frattini et al, 1997; Jianfeng et al, 2003). Based on the cell culture studies the function of NRF protein was defined as a constitutive inhibitor of NF-κB regulated promoters. To investigate the physiological function of NRF *in vivo* mice lacking NRF protein were created. This work describes the generation of these mice. By homologous recombination in mouse ES cells the encoding region of the endogenous NRF gene was replaced with a piece of recombinant DNA. The resulting locus still contains the NRF gene but half of the protein encoding the C-terminal fraction is flanked by loxP sites and the gene encoding neomycin resistance introduced into the 2nd exon of the NRF gene (Figure 2.1). By this gene interruption the expression of a truncated form of the NRF protein was achieved. This form contains a nuclear localization signal (NLS), the DNA binding domain (DBD) as well as the NF-κB repressing domain, whereas the nucleic acid binding domains G-Patch and R3H and consensus sequence motifs for RNA binding (JAG) are eliminated. Following blastocyst injection and breeding of chimeric mice, a colony of mice that carry the flanked NRF allele and express the "short" NRF was established. Expression of the flanked form of the NRF was confirmed by analysis of cellular mRNA with Northern Blots (Figure 2.15). Unfortunately, the existence of this short form of the NRF protein could not be proven because of the non-availability of a specific antibody. The expression level of mRNA corresponding to the flanked NRF is similar to that of the WT form. This let us expect normal translation and production of the truncated form of the NRF protein.

Mice with a complete deletion of the NRF gene (NRF-KO) were produced by crossing the NRF-flox animals with those expressing Cre recombinase. Upon recombinase action the flanked cassette including the major part of the NRF gene is excised, giving rise to mice completely lacking expression of the NRF protein. NRF absence was tested on different molecular levels: on the DNA level - with PCR, Southern Blot Analysis and sequencing; on the level of RNA – with RT-PCR and Northern Blotting; and on the protein level - by Western Blot analysis and Immunofluorescence. Since NRF-KO cells still contain the 5' UTR of the NRF gene, 1st exon and the distal part of the 2nd exon, transcription of this gene can be initiated and give rise to mRNA. This RNA cannot be processed by splicing because of the

lack of a splice acceptor (that was deleted together with the major part of the 2nd exon; Figure 2.16). Theoretically, its size is 3,9 kb. This was analysed with RT-PCR using intron- and 3'-end specific primers. The PCR product was found only in NRF-KO cells (Figure 2.30), correlating with the existence of the non-spliced form of the NRF mRNA in these cells. Thus, an mRNA is synthesized in NRF-KO cells that contains the 1st exon (115 bp), the 1st intron (1082 bp) and the distal part of the 2nd exon (870 bp). With Northern Blot analysis using a labelled 2nd exon specific DNA fragment no RNA band was detected in NRF-KO cells (Figure 2.17). This can be explained by a very low level of the intron-containing mRNA which might be due to its instability. Translation of NRF-KO mRNA into protein is unexpected because of multiple STOP codons in the intron part following right after the 1st exon.

Despite of the presence of a transcription product in NRF-KO cells no NRF protein was formed when tested with Western Blots using antibody directed against the C-terminus of the NRF which is encoded by the 3'-end of the NRF gene and is homologous to the NRF-KO mRNA (Figure 2.18). The presence of the peptide corresponding to the 1st exon of the NRF gene could not be tested. Because of the very small size (38 amino acids) a biological function of such a peptide is not very probable, but cannot be excluded. At least it would lack the so far defined functional domains of the NRF. Thus, a deletion of the NRF in NRF-KO samples and with this the generation of NRF-deficient mice could be confirmed.

NRF-KO mice are viable, appear phenotypically normal and exhibit normal life spans. It is demonstrated that NRF does not fulfill an essential function in mouse development or adult life. There are two possible explanations for this observation. First, NRF acts in NF-κB inducible promoters regulating genes involved in a variety of cellular processes. Most of the NF-κB regulated genes are silent in the constitutive situation. Upon extracellular stimulation these genes become activated and give rise to a rapid response. The best characterized promoters containing NRF binding sites are those of the cytokines IFN-β, IL-8 and iNOS. All these proteins in already low doses modulate serious biological effects and are therefore very tightly controlled. It seems, that NRF is not the only factor inhibiting the constitutive expression of these genes and that the absence of NRF can be successfully compensated by other factors. Dysregulation of the NF-κB system was shown to be involved in acute and chronic inflammatory processes as well as in cancer (Baldwin et al, 2001). Many human leukaemia's and lymphomas show constitutively active NF-κB in the nucleus. Also, the constitutive activation of the NF-κB pathway is often associated with inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis and asthma. The

elevated levels of NF-κB are accompanied by enhanced recruitment of inflammatory cells and the production of pro-inflammatory mediators. Although it is unclear whether increases in pro-inflammatory cytokine production are the cause or the result of NF-κB activation, constitutive NF-κB activity could be caused by defects in the regulatory mechanisms that control NF-κB activation. NRF is not the only factor preventing constitutive expression of NF-κB inducible genes. We assume that other regulators can functionally compensate NRF deficiency in NRF-KO mice. Such kind of a compensatory mechanism was already shown to exist in the NF-κB system. The activity of NF-κB is controlled at multiple levels. Extracellular signals activate degradation of the cytoplasmatic NF-κB inhibitor complex of IκB proteins and allows the translocation of NF-κB from the cytosol to the nucleus. The IκB proteins include IκBα, β, γ, ε, and ζ. Analysis of mice deficient in IκB members demonstrates that hyperactivation of NF-κB signaling has a dramatic impact on lymphopoiesis. Mice deficient in IκBα die about 10 days after birth and appear immunodeficient with extensive inflammatory dermatitis and secondary granulocytosis, due to a constitutive NF-κB activation (Beg et al, 1995). In contrast, IκBε-deficient mice exhibit a mild immunological phenotype, but demonstrate an upregulation of IκBα and IκBβ, suggesting that IκB members functionally compensate for each other (Memet et al, 1999). This hypothesis was confirmed through the generation and analysis of mice deficient in both IκBα and IκBε. In contrast to single IκBα or IκBε mutants, double mutant mice die at birth, lack mature B and T cells and their lymphocyte precursors exhibit increased apoptosis due to NF-κB hyper-activation (Goudeau et al, 2003). The same is true for differentiation of NK cells. Neither IκBα nor IκBε-deficiency have major effects on NK cell generation, while their combined absence leads to NF-κB hyper-activation, resulting in reduced NK cell numbers, incomplete NK cell maturation and defective IFN-γ production (Samson et al, 2004). The fact that the NF-κB system is redundant in its regulation highlights its biological importance and supports the hypothesis of NRF compensation.

Members of the IκB family of proteins keep NF-κB in the inactive form in the cytoplasm, but some level of the NF-κB proteins is present in the nucleus also in the unstimulated state. In this study we could confirm this fact testing nuclear extracts isolated from unstimulated cells with electromobility shift assays using an NF-κB specific probe (Figure 2.20). NRF acts on NF-κB silencing in the nucleus and does it by binding to the negative regulatory element (NRE).

Proteins that collaborate with NRF in the nucleus or compensate NRF absence are still to be identified. However, a number of repressor elements were recently described in the promoters of cytokine genes, similarly regulated by NF-κB. In the IL-1β promoter a novel negative element was identified that was suggested to play a role in suppressing its constitutive activity (Lebedeva et al, 1997). The promoter of the IL-6 gene contains a binding site for the mammalian transcriptional repressor RBP (CBF-1), which binding represses IL-6 activation (Vales et al, 2002). Despite a common function on the constitutive inhibition of NF-κB regulated promoters no apparent homology was found between these published sequences and NRE (table 3.1).

	Sequence	Found in promoters	References
NRE	**AATTCCTCTGA**	**IFN-β, iNOS, IL-8**	**Nourbakhsh, 1999**
negative element in IL-1β promotor	**AATATT**	**IL-1β**	**Lebedeva T., 1997**
RBP binding site	**TTTCCCATG**	**IL-6**	**Vales L., 2002**

Table 3.1. *Sequence comparison of the negative elements in cytokine promoters.*

IFN-β, iNOS, IL-8 similarly to IL-1β and IL-6 are not constitutively expressed in healthy tissues. Uncontrolled production of these cytokines may be harmful and contribute to pathological inflammatory processes. This is one of the reasons why regulation of cytokine genes has to be tight. And there is no evidence that different NF-κB inhibitory factors cannot replace one another in the case of functional inactivity of one of them, as for example, in the NRF-KO situation. This question is still to be addressed as well as the possible existence of additional repressing factors that can share their function with the known ones.

Murine NRF, similarly to the human homolog, is located in nucleoli. This is a cellular compartment where ribosomal RNAs processing and their assembly into ribosomes takes place. Analysis of the NRF protein showed that NRF can bind dsRNA (Niedick et al, 2004). The biological significance of nucleolar localization and RNA binding remains to be investigated. A classical example of a dsRNA-dependent protein is protein kinase PKR. PKR is an IFN-inducible gene that plays a role in antiviral host defense (Meurs et al, 1990). Following interaction with dsRNA, PKR becomes autophosphorylated and in turn phosphorylates target substrates, the best characterized being the α subunit of eukaryotic

protein synthesis initiation factor 2 (eIF2α) (Panniers and Henshaw, 1983). Moreover, PKR may provide a crucial first line of defense against certain types of viral infection by delaying the production of progeny virions through the inhibition of viral mRNA translation prior to the induction of IFN (Balachandran et al, 2000). In addition to this role, PKR has also been reported to function in a variety of signaling pathways, including those of dsRNA and NF-κB (Yang et al, 1995; Der et al, 1997; Kumar et al, 1997; Chu et al, 1999). Despite this evidence, mice lacking the PKR gene do not exhibit any significant pathology. Regarding immunity to viral infection in these mice it was shown that PKR deficient mice are predisposed to lethal VSV infections and display an increased susceptibility to influenza virus infection (Balachandran et al, 2000). Besides its ability to bind dsRNA it has been shown that NRF acts through DNA:protein interaction, binding to DNA (NRE), as well as protein:protein interactions (Nourbakhsh and Hauser, 1999). Whether all these functions are responsible for the constitutive repression of NF-κB regulated promoters or if NRF participates in infection, in the regulation of cellular responses remains unclear. A challenge of the NRF deficient mice with different bacterial and viral agents can clarify the question of the participation of NRF in the defence reaction of the immune system. In the case of a pure inhibitory role of the NRF the NRF-KO mice should be more resistant towards infection. However, when the NRF has a co-stimulatory function as well, some disturbance in the mechanisms of host defense is expected. Many immune responses are pathogen-type dependent. As mentioned above host response of the PKR deficient mice to a number of viruses including vaccinia virus were described as normal (Yang et al, 1995; Abraham et al, 1999) and this is in contrast to an increased susceptibility of these mice to VSV and influenza virus (Balachandran et al, 2000). In mice lacking a functional IFN-α/β receptor immune responses to a series of viral infections (Vaccinia virus, LCMV, VSV, Semliki Forest Virus) are impaired, whereas the resistance to L. monocytogenes infection is normal (van den Broek et al, 1995). Thus, additional studies are needed to elucidate a role of the NRF in host defense. In particular, *in vivo* infections of NRF deficient mice with series of different pathogens are needed.

3.2 Establishment and characterization of cells lacking NRF expression

For investigations on NRF function *in vitro,* NRF-KO cell lines were established. First, by *in vitro* excision of the NRF flanked cassette ES cells lacking the NRF gene were produced. These NRF-KO ES cells were then used for generation of differentiated endothelial cells by i*n vitro* differentiation. Using an earlier published protocol (Balconi et al, 2000) a population of

differentiated endothelial cells was obtained. For selective immortalisation of endothelial cells Polyoma virus middle T antigen (PymT) was used. Mice transgenic for the PymT oncogene show vascular tumors (Bautch et al, 1987) and retroviral constructs with PymT transform murine endothelial cells *in vivo* and *in vitro* (Williams et al, 1988). PymT, a membrane-associated protein, recruits and activates Src family tyrosine kinases, and once tyrosine is phosphorylated, binds proteins with PTB and SH2 domains such as ShcA, phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ-1 (PLCγ-1). PymT mimics activated receptor tyrosine kinases by forming a ShcA-Grb2-Gab1 complex that is critical for endothelial transformation (Siew Hwa Ong et al, 2001). Therefore, endothelial cells show a greater susceptibility to transformation with PymT as, for example fibroblasts, which require the expression of a second oncogene in addition to PymT for transformation (Rassoulzadegan et al, 1982). Although the cells described in chapter 2.3 do not have typical endothelial morphology they express endothelial specific markers like CD34 (Figure 2.15). Despite specific effects of the PymT on endothelial cells a homogenous population of endothelial cells could not be obtained. It is proposed that for elimination of non-endothelial cell types further culture of the PymT infected cells is needed.

Two other types of primary cell culture were used for further molecular studies: murine fibroblasts and macrophages isolated from NRF-KO mice. It was proposed that the absence of NRF causes some level of constitutive activation of the promoters in which regulation NRF is involved. Fibroblasts known as a major producer of IFN-β were analysed for constitutive expression of this cytokine. Macrophages were tested with respect to the activation of the iNOS promoter. Neither fibroblasts nor macrophages had acquired the expression of the tested cytokine genes in the non-induced state. Upon induction (NDV for fibroblasts and LPS for macrophages) both cell types reveal normal regulation and expression levels of IFN-β and iNOS, respectively (Figures 2.22 and 2.24). Unfortunately, the situation in cell culture does not always reflect activities in the whole organism. The two cell types examined here are only components of the complex immune response machinery. We have not examined the effects of NRF absence on other cells of the immune system, although NF-κB plays essential roles in the maturation and differentiation of T and B cells. Moreover, these cells show some constitutive NF-κB activity in certain maturation stages.

Since NRF is expressed already during embryogenesis (Schwarzer, 2001) it is suggested that NRF deletion in the early stages is developmentally compensated. To examine this

compensation process a method for gene deletion in primary cells was established. Efficient retroviral infection was used for transduction of primary cells. Cells homozygous for the NRF-flox allele were infected with virus carrying a gene encoding Cre recombinase. Upon Cre action the flanked NRF gene was excised giving rise to NRF-KO cells. The use of Cre bearing retrovirus for deletion of the flanked cassette in cultured cells was already applied in previous studies (Silver et al, 2001). But in the current work some modifications were undertaken. First, Cre was fused to GFP protein. This allows to isolate cells that have undergone Cre mediated gene excision latest 72 hours after infection. Because of the long duration and toxicity of commonly used drug selection the advantage GFP-Cre fusion protein is especially useful for studying of rapid effects caused by the gene deletion. Secondly, in contrast to other studies this work was carried out on cell culture of freshly isolated primary cells. It was shown that GFP-Cre encoding retrovirus can be successfully used for sudden deletion of the flanked gene in primary cells. Although the infection of primary cells was not as efficient as of NIH3T3 cells, sorting of GFP positive cells resulted in a homogenous population of cells that had undergone gene excision. These KO cells were further propagated and analysed. Primary fibroblasts carrying the NRF-floxed allele were subjected to this procedure. The expression of the IFN-β gene in NRF-KO cells was analysed. Unfortunately, no constitutive activation of the IFN-β upon sudden deletion of the NRF was detected. Thus, deletion of the NRF alone (early in embryogenesis or sudden in adult cells) does not provoke an induction of the IFN-β promoter. This is in contrast to previous study where NRF was inactivated using NRF antisense RNA (Nourbakhsh and Hauser, 1999). This discrepancy can be explained by 1) a difference in the time course between Cre-mediated deletion of the NRF and the activation of the IFN-β gene, and 2) specific properties of the tested cells, as for example, some increased level of NF-κB in the nucleus of the human HeLa cells (these cells were used for antisense experiments) versus normal levels of NF-κB in the fibroblasts used in this study. Data of the current study indicates the presence of one or more factors that function on the inhibition of basal activity of this cytokine to compensate the lack of NRF.

3.3 Effects of NRF deficiency and protein overexpression on the gene expression pattern

To determine candidate genes which expression is affected by changes in the NRF expression an expression profiling was carried out. This assay can also help to recognize genes that indirectly interact with or are regulated by NRF. To reveal thorough information from this assay cells lacking NRF expression as well as NRF overexpressing were analysed. The

numerous genes which expression was somehow affected were organized in 10 clusters. Each cluster contains genes with similar expression pattern. Since regulated genes belong to different groups and are involved in various cellular processes no direct influence of NRF on a defined pathway could be found. Even genes participating in cell metabolism were amongst the regulated genes. Analysis of the expression patterns revealed some interesting facts:

1) There are genes which expression is affected by NRF deficiency. This effect includes both up- and down-regulation of genes.
2) There are genes affected only by sudden excision of the NRF gene. The expression of these genes in developmentally produced NRF-KO animals is similar to the level of their expression in WT cells. In this way regulated genes are schematically presented on the Figure 2.37. Cluster B groups the genes which expression was down-regulated upon sudden deletion of the NRF. While in the cluster H genes are collected that were induced by the sudden NRF knock-out. This let us confirm that there must be some adaptive activity which serves to compensate the deletion of the NRF gene when deletion occurs during early stages of embryogenesis. Adult cells are not competent any more for this kind of adaption.
3) NRF overexpression also has both, inducing and repressing effects on the different genes (Figure 2.37; sample 4, clusters C, E and I).

Detailed investigation of genes and signalling pathways involved in the compensatory process is matter of future work. An analysis of genes affected by the expression of the NRF let us propose a role of this protein in the regulation of apoptosis. In this model NRF deficiency is associated with reduced apoptosis, as a result of inhibition of CytC and caspase3 genes and induction of the apoptosis inhibitor Mdm2, while NRF overexpression leads to up-regulation of the some proapoptotic genes like p53 (Figure 2.40). Involvement of the NRF protein in the regulation of apoptosis was already proposed previously (Schwarzer, 2001). It was shown that during day 12 in murine embryogenesis NRF is strongly expressed in the interdigital web. The first sign of NRF expression in this area was found at day 11,5 of embryogenesis, while from day 12,5 the expression was maximal. This stage of development is associated with the formation of the paws and separation of the digits as a result of directed cell death in interdigital space. Thus, it was speculated that strong NRF expression can be correlated with a high level of apoptosis. A current study tries to confirm this hypothesis. Thus, the molecular mechanism of NRF action in the apoptosis process is to be clarified. NF-κB that appears as a main subject of the NRF inhibitory action promotes cell survival by initiation of the

transcription of antiapoptotic genes, such as Bcl-2, cIAP1 and cIAP2 (Chen et al, 2000; Wang et al, 1998).

It was demonstrated that NF-κB has a critical role in cell cycle progression. The NF-κB activity was elevated during the G_0-to-G_1 cell cycle transition in mouse fibroblasts (Baldwin et al, 1991). The link between NF-κB activation and the control of the cell cycle progression was also found in human HeLa (Bash et al, 1997), Jurkat (Perkins et al, 1997) and human glioma cells (Otsuka et al, 1999). The NF-κB binding sites were identified in the promoter region of the cyclin D1 gene (Joyce et al, 2001) that provides direct evidence of the involvement of NF-κB in cell cycle regulation. Cyclin D1 in association with cyclin-dependent kinases (CDKs) promotes G_1-to-S-phase transition through CDK dependent phosphorylation of pRb and activation of transcription factor E2F, which is required for the activation of S phase-specific genes. Elevated expression of the NF-κB family of proteins is associated with oncogenesis. It was shown that 1) c-Rel induces aggressive leukemias/lymphomas in chickens and transgenic mice (Gilmore et al, 1999); 2) inactivation of RelA protein in mice results in massive liver cell apoptosis (Beg et al, 1995) 3) few alterations in the structure and expression of p50/p105 subunit of the NF-κB have been reported in leukemias and lymphomas (Liptay et al, 1992). The possible mechanism of dysregulation of the NF-κB expression is an impaired function of NF-κB inhibitors. An important inhibitor of the NF-κB is IκB. Recent analysis of many human tumors demonstrate persistant nuclear NF-κB as a result of defective IκB activity. In this context it could be proposed that NF-κB inhibitors act as a tumor supressors to control the oncogenic activation of NF-κB. Moreover, there is evidence that suggest that overexpression of the NF-κB may prevent the efficiency of chemotherapy and lead to chemoresistance. It has been shown that the inhibition of NF-κB by PS-341 markedly increases the sensitivity of myeloma cells to chemotherapeutic agents (Berenson et al, 2001). Thus, inhibition of the NF-κB pathway using natural NF-κB inhibitors including NRF may represent a novel strategy and lead to successful therapy with existing chemotherapeutic agents.

3.4 Summary and future perspectives

In the presented work the study of the role and the function of the NRF was undertaken using a model of transgenic mice expressing mutant forms of the NRF protein. It was shown that lack of the NRF alone does not lead to constitutive activation of NRF regulated genes in

tested cells. It was proposed that because of the tight regulation of the cytokine genes, NRF deficiency must be compensated. Investigation of collaboratory partners of the NRF and the molecular mechanism of its inhibitory action is an important aspect of the future studies. While sustained cytokine overproduction that is typical for some diseases has detrimental effects, including cellular injury and apoptosis, understanding the mechanism of basal repression of the NRF target genes might lead to a new strategy for tumor therapy.

To elucidate a full role of the NRF in the mechanism of the regulation of the immune-relevant genes immune defense of the mice lacking the NRF protein should be analysed. The NRF-KO mice will be challenged with different bacterial and viral pathogens. This work needs a big animal resource. Currently preparatory work for this kind of experiments is carried out. Analysis of efficiency of the immune response in NRF deficient mice will reveal whether NRF has only inhibitory function or it has as well any co-activatory role. This information will provide insights into the role of the NRF in the mechanism of the host defense.

4. Materials and Methods

4.1 Chemicals

chemicals were supplied by the companies Amersham, Bayer, Biolabs, Biorad, BRL Difco, Gibco, Merck, Miles, Prenner, Roche, Pharmacia, Qiagen, Seromed, Serva and Sigma. Enzymes were purchased from Biolabs, BRL, Merck, Perkin-Elmer, Promega, Roche, Serva and USB. Antibodies were obtained from Pharmingen, Silenus, Dianova, Clontech and IBGRL. Oligonucleotides were purchased from the companies Gibco and MWG. DNA-sequencing was done by DNA-synthesis-group of the GBF or by MWG. Radioactive chemicals were purchased from Amersham-Buchler.

4.2. Mice

Specific pathogen-free C57BL/6 mice (4 weeks old) were purchased from Jackson laboratories.

NRF-lox chimeric mice were generated at Mice&More laboratories (Hamburg) by injection embryonic stem cells from a strain 129/Ola embryonic stem cell line with a targeted mutation in the NRF gene into a C57BL/6 blastocyst.
Identified chimeric mice were outcrossed to C57BL/6 for strain maintenance.

Mice with a complete deletion of the NRF gene (NRF-KO) were produced by crossing the NRF-flox animals with expressing Cre recombinase K14Cre mice.
NRF-lox and NRF-KO mice were maintained on a mixed C57BL6 x 129 genetic background. Animal colonies are kept in pathogen free conditions at the Animal Facility of the GBF.

K14Cre mice were kindly provided by Dr. Hafner (Department of Experimental Immunology, GBF, Braunschweig).

4.3. Eukaryotic cell culture

Culture media and solutions:

All reagents were dissolved in “Millipore”-water and sterile filtrated (pore size of 2 μm).

Pen/Strep (100x):	1.212 g Pennicillin/200 ml (10000 U/ml) 2 g Streptomycine/200 ml (10 mg/ml) to dissolve adjust to pH 7.0 with NaOH, stored at -20°C
PBS:	140 mM NaCl 27 mM KCl 7.2 mM Na_2HPO_4 14.7 mM KH_2PO_4 (pH 6.8 - 7.0)
TEP:	500 ml sterile PBS 0.6 ml 0.5 M EDTA (final conc.: 6 mM) 15-20 ml Trypsine (0.1 - 0.2%; depending on the activity)
Puromycin:	5 mg/ml in H_2O, sterile filtered, stored at - 20°C
G418:	100 mg/ml in H_2O, sterile filtered, stored at - 20°C
Hygromycin:	431660 U/ml (batch dependent), sterile filtered, stored at 4ºC
HEBS (2x):	280 mM NaCl 50 mM HEPES 1.5 mM Na_2HPO_4 (pH 7.1)

Gelatin (2% Sigma, cell culture grade)

Medium:	Dulbecco's modified Eagle's medium (Sigma) RPMI

Cells:

ES- E14TG2a, strain 129/Ola

This is a derivative of one of several murine embryonic stem cell (ES) lines developed by M. Hooper in 1987 (Smith and Hooper, 1983). The cells are deficient in HGPRT (HPRT) and are resistant to 0.06 mM 6-thioguanine. The cells remain undifferentiated when cultured on feeder layers (embryonic fibroblasts). The cells spontaneously differentiate and form embryonal structures in the absence of a feeder layer. When injected into blastocysts, the cells can colonize the germline.

Medium: Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 0.1 mM 2-mercaptoethanol, 90%; fetal bovine serum, 10%.

MEF- Murine Embryonic Fibroblasts

For long-term culture and maintenance, pluripotent ES cells must be grown on feeder cells. We have used monolayers of mitotically inactivated primary embryonic fibroblast cells. These cells were prepared from mouse embryos on days 11.5-12.5 of embryogenesis. To stop proliferation the cells were irradiated with 3000 rads of gamma irradiation.

Mouse skin fibroblasts

Primary dermal fibroblasts were isolated from 2-day-old mice. Trunk skin was removed, washed 1x in antiseptic Betaisodona Solution, 1x in75% ethanol and then in sterile phosphate-buffered saline (PBS) and incubated overnight at 4°C in 0.25% trypsin in order to strip off the skin at the dermo-epidermal junction. After this enzymatic treatment, the epidermis was then mechanically separated from the dermis, the epidermis was peeled off and discarded. The resulting dermis layers were treated with 0,15% Collagenase A for 30min at 37°C and then mechanically dissociated. The digested dermal cell suspension was filtered through a sterile 150 μm stainless steel mesh filter to remove debris. Thereafter the cell suspension was centrifuged for 5 minutes at 1200 rpm to achieve a single dermal cell pellet. This cellular

pellet was suspended and cultured in DMEM supplemented with 10% FCS. The culture medium was changed every two or three days.

Resident peritoneal macrophages

Mice were sacrificed and peritoneal macrophages were obtained by peritoneal lavage with cold phosphate buffered saline (PBS). 8 mL of sterile PBS was injected into the peritoneum and withdrawn. The cells from 2-5 mice were then combined, centrifuged at 1200 rpm and pelleted cells resuspended in serum-free RPMI 1640 medium. The cells were then plated in plastic tissue culture dishes (Corning), incubated at 37°C for 2 h, and washed three times with PBS to remove unattached cells. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum.

Cell lines:

NIH3T3 - embryonic mouse fibroblast cells, ATCC-CRL 1658

LMTK- thymidine kinase-deficient mouse fibroblast cells (ATCC No. CCL 1.3)

Mouse C243 cells – (Oie et al, 1977)

GP+E86 - ecotropic RV packaging cells (Markowitz et al., 1988)

In vitro differentiation of ES cells

The relevant steps of cell isolation and culture are indicated in Figure 2.12. ES cells were grown in the undifferentiated state either on a feeder layer of irradiated murine fibroblasts. To induce ES differentiation, LIF and feeder cell layers were omitted, and ES cells were cultured in the presence of a cocktail of endothelial cell growth factors. Cells formed embryoid bodies, which were then collected and disaggregated in a collagenase-DNase solution by gentle pipetting. Endothelial cell lines were then developed by direct infection with PymT carrying retrovirus.

The culture medium of undifferentiated ES cells was DMEM with 10% FCS supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 1000 U/mL LIF, 1 mmol/L sodium pyruvate, and 0.1 mmol/L MEM nonessential amino acid.

To initiate ES cell differentiation and EB formation, ES cells were briefly trypsinized and suspended in DME medium. Vascular differentiation was optimized by the addition of a growth factor cocktail to the culture medium: recombinant human VEGF (Peprotech Inc) was used at 50 ng/mL, human FGF at 100 ng/mL. Cells were seeded in bacteriological Petri dishes (1.5×10^4 cells per 35-mm Petri dish) and cultured for 11 days, without further feeding, at 37°C in an incubator with 5% CO_2 in air and 95% relative humidity. The cells formed EBs. EBs at E11 were collected and disaggregated by 1-hour incubation in 1.5 mg/mL collagenase A (Boehringer-Mannheim) in DMEM plus 5% FBS. After 50 minutes of incubation, 25 µg/mL DNase (Boehringer-Mannheim) was added.

After EB disaggregation, the cells were immortalized by PymT. Cell immortalization was performed as following. 24 to 48 hours after seeding in 24-well plates (0.5 to 1×10^6 cells per well), the cells were incubated with $\approx 10^5$ puromycin-resistant colony-forming units of the retrovirus vector N-TKmT in 1 mL of complete medium per well in the presence of 8 µg/mL polybrene (Sigma). The virus-containing medium was replaced 3 hours later with fresh complete medium. 72 hours later, PymT-infected cells were selected by adding the puromycin at 2,5 µg/mL. Fresh medium containing puromycin was replenished 3 times per week. Colonies of puromycin-resistant cells, were observed after 20 to 30 days.

The culture medium of ES-derived endothelial cells was DMEM with 10% FCS, supplemented with 2 mmol/L glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL endothelial cell growth supplement (Sigma), and 100 µg/mL heparin (Sigma).

4.4. Cell induction and cytokine detection

Infection with NDV

Cell were washed 2x with DME medium without FCS before infection and then incubated with NDV containing (1:8000 dilution) DMEM without FCS for 1h at 37°C. After that cells were washed 3x with FCS containing medium and incubated in normal growth medium (10%

FCS) for futher 24h. Supernatants were then collected and tested in IFN-test. Cells were used for DNA isolation or discarded.

IFN-test

The IFN concentrations in the cell culture supernatants were determined by an antiviral assay based on the inhibition of the cytopathic effect of vesicular stomatitis virus (VSV) on IFN-sensitive LMTK$^-$ cells (Dinter and Hauser, 1987).
LMTK$^-$ cells (1,3 x 10^4 cells in 100 µl of DMEM containing 10% FCS) were plated per well in a 96-well plate. 24h later two-fold serial dilutions of test culture supernatants or a standard IFN-β were added. Following incubation for 24 h at 37°C, the supernatant of each well was aspirated. Each well then received 100 µl of VSV suspension containing approximately 40 plaque-forming units/ml. The plates were incubate at 37°C. Protection was evaluated at 24-48 h postchallenge by visual examination. Remaining cells were then stained with 1% crystal violet.

Induction of macrophages with LPS

24h after plating macrophages were stimulated with LPS (from *Escherichia coli,* Sigma-Aldrich) by incubation with indicated in each experiment concentration of LPS diluted in normal growth medium.

Detection of NO production

Supernatants were collected from the induced and control cells and analysed. The accumulation of NO_2, a stable product of NO metabolism was measured as an indicator of NO production. 100 µl cell-free medium was incubated for 30 min with 100 µl Griess reagent (1% sulfanylamine in 30% acetic acid, and 0,1% N-(1-naphthyl) ethylenediamine dihydrochloride in 60% acetic acid; 1:1, v/v; Sigma) at room temperature and the absorbance at 540 nm was measured in a microplate reader. The NO_2 concentration was calculated from a standard curve of sodium nitrite.

4.5. Gene transfer methods

Electroporation of ES cells

The day before transfection the ES cells were plated on 100 mm gelatin coated dishes. For electroporation, the cells were trypsinized, washed with PBS and centrifuged. The cell pellets were resuspended in 1ml PBS (up to 10^7 cells) and pippeted into an electroporation cuvette together with 20 µg DNA. Electroporation was carried out at 220 V and 500 µF. After that the cells were plated on mitotically inactivated MEFs in ES growth medium.

Transfection with calcium-phosphate

Stable transfection (according to Pellicer et al., 1978):
One day before transfection the cells are seeded in 6-wellsplate (2-5 x 10^4 cells/ well). 4 hours before transfection fresh DME medium is put on the cells. 5 µg of the DNA to be transfected is suspended in 200 µl 250 mM $CaCl_2$. The solution is added drop by drop to 200 µl 2x HEBS buffer under continuous vortexing. In this solution the DNA coprecipitates with the calcium phosphate solution for 5-10 min Then the precipitates are pipetted to the cells and 15 hours later the medium on the cells is replaced by fresh medium. 2 days after transfection selection of stably transfected cells is started. The cells are transferred to a 55 cm^2 plate or a 75 cm^2 flask and are selected.. Medium exchange is done every 3 to 5 days. The selection is finished in general after 7-12 days.
The concentrations used for each selective drug is dependent on the cell type and has to be tested in advance. In this work following concentrations were applied:
G418 – 1000 µg/ml
Hygromycin B – 0,2 µg/ml
Puromycin – 2,5 µg/ml

Transient transfection:
The preparation of the precipitates is identical to the method described for stable transfection but in this case there is no need for a selection because the expression from the extra chromosomal DNA (circular plasmid) is measured.
At day one after transfection the precipitates are removed by medium exchange. At day two the cells can be analysed for the expression of the transfected gene.

Retroviral infection of mammalian cells

Establishing virus producer cells:
Ecotropic packaging cells GP+E86 were plated in the concentration 8 x 10^4 cells / well of 6wells-plate. On the next day these cells were transfected with retroviral plasmid using standard calcium-phosphate method. After 7-10 days of selection viral titer in the supernatant can be determined.

Infection:
Packaging cells with a relatively high titer (10^4-10^6 CFU/ ml) are seeded in such a way that they are subconfluent at the day of infection. Packaging cells with a much lower titer can be grown to confluency at the day of infection. The DME medium on the packaging cells is renewed on the day before infection (virus production medium). The production medium should stay on the producer cells for at least 24 hours. On the same day the cells are seeded which have to be infected in a concentration of 2 x 10^4 cells / well of 6wellsplate. On the next day the supernatant (virus containing medium) of the producers is harvested, mixed with polybrene (8 µg/ml final concentration), filtered (0.45 µm filter) to eliminate cells in the supernatant and given to the cells for infection in wanted dilutions (in polybrene containing medium; polybrene increases the efficiency of the infection through a better virus uptake). 24h later medium is changed to the normal growth medium.

Titer assay:
For the determination of the viral titer NIH3T3 cells are seeded into a 24-well plate (5000 cells/well). On the next day dilutions of the virus containing medium up to 10^{-6} are added after the medium on the 24 wells was exchanged for polybrene containing medium. For an exact virus titer determination the number of producer cells per ml production medium should be counted. 24-48 hours after infection the medium is exchanged for selection medium: The appearing cell clones can be stained with crystal violet at day 7-12 after the infection.

Electrotransformation of DNA into *E. coli*

For the electrotransformation a mix of competent cells and DNA is exposed to a exponentially decreasing electric field with a very high start amplitude. The transformation efficiency is

situated at 10^9-10^{10} transformants per µg of DNA depending on the quality of the electrocompetent bacteria. A 50 µl aliquot of competent cells is thawed at room temperature and put on ice. 1 µl of plasmid DNA or ligation mix is added, mixed thoroughly and this mix is pipetted into a sterile pre-cooled 0.2 cm electroporation cuvette. The cuvette is exposed to an electric field in a electro-transformation room. Pulse controller and gene pulser are set at 2.5 kV, 25 µF and 200 Ω. The time constant should range between 4 to 5 ms. Immediately after transformation 1 ml LB-medium is added to the cuvette. The transformed cells are shaken 30 min at 37°C and subsequently are plated out in different dilutions on agar plates containing the appropriate selection medium.

4.6 DNA analysis

Extraction of Genomic DNA

A 1 cm mouse tail snip or cell pellet was incubated with 300 µl lysis buffer containing 1 mg/ml Proteinase K at 54°C overnight until tissue is completely digested. The lysats were spinned down at maximal speed for 30 minutes. The supernatant was transferred to a new tube. For DNA precipitation the equal volume of isopropanol was added and mixed by gentle invertion. The DNA pellet was obtained after centrifugation at 13000 rpm for 15 min. The DNA pellet was washed with 70% ethanol, dried and resuspended in TE buffer.

Lysis buffer: 50 mM Tris
0.4 M NaCl
100 mM EDTA
0.5 % SDS

Boiling prep method (according to Dan Cimbora, FHCR, Seattle, USA)
This method is used for DNA isolation from *E. coli*. The major advantages of this method are the quick preparation and the high quality of the isolated DNA (based on 'Boiling prep' from Holmes and Quigley).

STET: 8% sucrose
0.5 % Triton
50 mM EDTA
10 mM Tris (pH 8)

LTE: 10 mg lysozyme/ 1 ml TE
7.5 M NH_4OAc

TER: 10 µg RNase A/ 1 ml TE

From the clones that have to be screened 2 ml cultures (LB+Ampicillin) were prepared and incubated in Kapsenberg tubes overnight at 37°C. After transferring the probes in 2.2 ml Eppendorf tubes, they were centrifuged for 1 min at 5000 rpm. The pellets were resuspended in 500 µl STET buffer and per tube 50 µl LTE was added. After a short incubation of 2-3 min at RT the probes were heated for 90 sec in an Eppendorf thermomixer at 95°C. After 5 min centrifugation (14000 rpm) a pellet is formed, which can then be removed with a toothpick. Then 50 µl 7.5 M NH_4OAc and 500 µl isopropanol are added; the mix is vortexed and the DNA is pelletted by centrifugation for 5 min at 14000 rpm. After drying for a short time the pellet is solved in 100 µl TER solution. For restriction analysis 5 µl is used.

Random priming with the "Redi-Prime DNA labeling system"

Labelling mix:	dATP, dCTP, dTTP, exonuclease free Klenow enzyme, oligonucleotide primer (9-mer)

For this labeling reaction a commercially available system of the company Armersham is used. The reaction mix is available as a freeze-dried pellet in an Eppendorf tube. First 2.5-100 ng DNA probe in 45 µl sterile water is denatured at 95°C for 5 min and put on ice immediately. The DNA and 5 µl of $\alpha[^{32}P]$dCTP are added to the labeling mix, vortexed thoroughly and incubated for 10 to 30 min at 37°C. To stop the reaction 2 µl of 0.5 M EDTA is pipetted to the mix.
After labeling, the probe is separated from unincorporated nucleotides over a Sephadex G50 column. For this purpose the ready to use stacked Micro-SpinTM G50 columns from the firm Pharmacia Biotech are used. The perfect packing of the column is reached by spinning the columns for 1 min at 2800 rpm. The labeling mix is put on the column and centrifuged through the column for 2 min at 2800 rpm. The labeled probe is denatured in a boiling water bath for 5 min, put on ice immediately and added to the hybridizing solution.

Southern Blot Analysis

For Southern, 10 µg of genomic DNA was overnight digested with the appropriate enzyme and run on 0.7% agarose gels in TAE solution. DNA was transferred to positive charged Nylon membrane in 0,4M NaOH and cross-linked to the membrane by drying at 80°C for 2h.

Southern blot hybridisation was performed using standard techniques. Hybridizations were done in buffer consisting of 0,5 M $Na_xH_yPO_4$, pH 7,2, 7% SDS and 2mM EDTA.
The membrane has to be pre-hybridized for at least 30 min and then the denatured radioactively labeled probe is added to the tube and hybridization takes place overnight at 68°C. After hybridisation the membrane is washed with the following set of washes:

2x 5 minutes with	Wash buffer I:	40mM $Na_xH_xPO_4$, pH 7.2 1% SDS 2 mM EDTA
1X, 20 minutes with	Wash buffer II:	40mM $Na_xH_xPO_4$, pH 7.2 0.1% SDS, 2mM EDTA
3X, 10 minutes with	Wash buffer III:	100 mM $Na_xH_xPO_4$, pH 7.2 2 mM EDTA

The washes are carried out at 68°C while shaking.

Re-hybridisation:

For rehybridization, the probe is eliminated by washing several times in 0.1% SSC/ 0.1% SDS at 95°C. To check if the probe is completely removed, the stripped blot is exposed again. Rehybridization is performed as described previously.

Polymerase Chain Reaction (PCR)

The PCR is a method used for amplification of defined DNA fragment using specific primers (approx. 20 bp long) that recognize appropriate sequences in the leading and lagging strand of DNA. PCR will allow a short stretch of DNA to be amplified to about a million-fold so that one can determine its size, nucleotide sequence, etc. There are three major steps in a PCR, which are repeated for 30 or 40 cycles:

1. Denaturation

   During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop (for example: the extension from a previous cycle).

2. Annealing

   In this step primers anneal to the DNA and the polymerase can attach and starts copying the template.

3. Elongation

   This is the ideal working temperature for the polymerase. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template)

PCR reaction components:

PCR polymerase Buffer
dNTPs mix (25 mM each nucleotide)
primer mix (25 pmoles/µL each primer)
Taq DNA polymerase (1 Unit/25 µL)
genomic DNA template (100 ng/µL)
the reaction will be adjusted with water up to 50ul volume.

PCR programm used for amplification of NRF fragments:

First denaturing	94 o C	10 min
Denaturing	94 o C	30sec
Annealing	62°C	30sec
Elongation	68°C	4 min
Last elongation	68°C	10 min

Afterwards the PCR products are run on 1% agarose gels.

DNA-isolation from agarose gels with‘Qiaquick’ gel extraction kit

Under high salt conditions the DNA-agarose piece is melted at 50°C for 10 min The resulting solution is centrifuged for 1 min through a mini-column. In this step the DNA binds to the column matrix, while the agarose-salt solution is running through. The column is washed with an ethanol-containing buffer. The DNA is eluded from the column with 30 to 50 µl TE.

4.7 RNA analysis

Isolation of total cellular RNA

Total cellular RNA was isolated using Trizol reagent. The cell were trypsinised, washed with cold PBS and spun at 1000 rpm at 4°C. 1 ml Trizol reagent was added to each cell pellet. The cells were mechanically lysed by passing the cell lysate several times through a pipette. 0.2ml chloroform per 1ml of TRIZol reagent was added to each sample. After vigorously mix and centrifugation at 13000 rpm 15 min at 4°C two phases are separated: lower red phenol-chloroform layer, interphase and colourless upper RNA phase. For RNA precipitation aqueous, upper clear RNA containing phase was transferred to a new tube, 0.5 ml isopropanol was added, mixed and centrifuged at 13000 rpm, 4°C for 15 min. RNA pellet was washed with 75% ethanol, air dried and redissolved in RNase-free water.

The concentration of the RNA was determined by measuring the A260 of the final preparation. A solution whose A_{260} = 1 corresponds to approximately 40 µg of mRNA per ml. The ratio between the readings at 260 nm and 280 nm gives an estimate of RNA purity. An A_{260}/A_{280} absorbance ratio in the range of 1.8 to 2.0 indicates a pure preparation of RNA.

Purification of mRNA

The Dynabeads® mRNA Purification Kit is based on the unique Dynabeads magnetic separation technology. The use of Dynabeads mRNA Purification Kit relies on base pairing between the poly A residues at the 3' end of most messenger RNA and the oligo dT residues covalently coupled to the surface of the Dynabeads Oligo $(dT)_{25}$. Other RNA species lacking a poly A tail will not hybridise to the Dynabeads Oligo $(dT)_{25}$ and are readily washed off. The mRNA is captured by the Dynabeads Oligo $(dT)_{25}$ and washed thoroughly using a magnet (Magnetic Particle Concentrator, Dynal MPC®). The mRNA is eluted from the solid phase by using a low-salt buffer. As starting material for the purification of mRNA we are taking 75 µg of total RNA. Initially the RNA should be heated to 65°C for 2 minutes to disrupt secondary structures. Annealing of the mRNA to the Dynabeads Oligo $(dT)_{25}$ is carrying out in binding buffer by rotating on mixer for 3-5 minutes at room temperature. After that the tube is placed on the magnet and the supernatant is pipetted off. After two washes with washing buffer, the

mRNA is eluted in 10ul of 10 mM Tris-HCl by heating to 80°C for 2 minutes. Eluted mRNA is transferred to a new RNase-free tube and stored at -80°C.

Binding Buffer - 4 ml
20 mM Tris-HCl (pH 7.5)
1.0 M LiCl
2 mM EDTA

Washing Buffer B - 4 ml
10 mM Tris-HCl (pH 7.5)
0.15 M LiCl
1 mM EDTA

RT-PCR

RNA was reverse transcribed using the SuperScript™ First-Strand Synthesis System for RT-PCR (Gibco). Total RNA (1-5 μg) was combined with oligo-dT primer mix, dNTP mix and H_20 and preheated at 65°C for 2 min to denature secondary structures. To the mixture was then added 5x RT Buffer, 10 mM DTT and RNase inhibitor. After 2min incubation at 42°C Reverse Transcriptase was added to the samples. The RT reaction was incubated at 42°C for 50 min following by heating at 95°C for 5 min. To remove RNA complementary to the cDNA the samples were treated with 1 μl (2 units) of *E. coli* RNase H at 37C for 20 min. The cDNA can now be used as a template for amplification in PCR.
A control experiment without reverse transcriptase was performed for each sample to verify that the amplification was not caused by any residual genomic DNA. The mRNA for GAP-DH was examined as the reference cellular transcript in each PCR reaction.

Northern blot analysis

Northern blotting is a method for RNA analysis. Especially significant is to study how cells express different levels and types of mRNA. In this method RNA is loaded on denaturing 0.8% agarose gels containing 1.2% formaldehyde and run overnight by 26 V. Next, the RNA is transferred onto a nylon membrane by capillary action in highly concentrated salt solution 10xSSC. Once transferred, the RNA should be covalently cross-linked onto the membrane, by heating at 80°C for 2h. Detection of a specific mRNA in the total RNA sample is done by hybridization of the Blot with a specific, complementary radiolabelled DNA probe overnight at 65°C. This hybridization step followed by autoradiography.

Agarose gel : 1.2 g agarose,
10 ml 10×MOPS buffer,
18 ml formaldehyde (12,3M)

10×MOPS buffer: 200 mM MOPS,
50 mM Sodium acetate,
10 mM EDTA, pH 7.0

Running buffer : 100 ml 10×MOPS buffer

Hybridization buffer: 10% Dextransulfat
1% SDS
1 M NaCl

0,5 mg/ml Yeast-RNA
0,5 mg/ml salmon sperm DNA

5×GLB: 16 µl bromophenol blue (1mg/ml),
80 µl 500mM EDTA, pH 8.0,
720 µl formaldehyde,
2 ml 100% glycerol,
3084 µl formamide
4 ml 10×MOPS buffer

Post-hybridization washes were done as follows:

2x, room temperature, 20 minutes, 2x SSC/1% SDS
1x, 65° C, 20 minutes, 2x SSC/0.5% SDS
1x, 65° C, 20 minutes, 0.1x SSC/0.1% SDS

Gene expression analysis

Microarray hybridizations were performed by the Array facility, GBF, Braunschweig. Affymetrix GeneChip® Murine Genome U74Av2 arrays were used which consist of coated glass slides with series of oligonucleotide probes synthesized *in situ*. These arrays contain probes for functionally characterized genes and EST clusters. Biotinlabeled cRNA probes were generated from each sample to be analyzed, starting form 5 µg of DNAseI-treated total cellular RNA, prepared as described above. The cRNA probes were individually hybridized on the arrays and the signals were detected according to the manufacturer's instructions (Affymetrix). Hybridization data were analyzed using the MAS5.0 (Affymetrix) software. Genes were considered as significantly expressed in a given experiment, if they were classified as P (present), but not as M (marginal) or A (absent).

4.8 Protein analysis

Preparation of nuclear extracts

Nuclear extracts were prepared using NucBuster Protein Extraction Kit (Novagen) and following manufacture protocol. After trypsinization of adherent cells the cell pellet was washed with PBS and NucBuster Reagent 1 was added (150 µl per 50 µl packed cell volume). After vortexing and 5 min incubation on ice the cell lysets were cetrifuged and supernatant, corresponding cytoplasmic fraction, was removed. The pellet were then resuspended with NucBuster Reagent 2 containing Protease Inhibitor Coctail and reducing reagent DTT. After additional vortexing and incubation on ice, the lysat was centrifuged. The supernatant – nuclear extracts was transferred to a separete tube and stored in aliquots at -80°C. The pellet left after nuclear protein extraction was used for additional nucleolar protein extraction.

BCA assay

This method was used for measurement of the protein concentration.

Reagent A: 1% Bicinchonin acid (BCA)
0.16 M Na_2CO_3
7 mM Na-tartrate
0,11 M $NaHCO_3$
1 ml 1 N NaOH
fill up to 100 ml with H_2O; (pH 11.25)
Reagent B: 4% $CuSO_4$ x 5 H_2O
Work solution: 10 ml reagent A + 0.2ml reagent B

In a 96-wellsplate the wells of the first row are filled with 190 µl H_20, in all other wells 100 µl/well is added. In the first well of the first row (A1) 10 µl of Triton lysis buffer is pipetted, in A2 10 µl of reference protein solution (3 mg/ml lysozyme) are put and 10µl of each probe are added to the first row. With a multi-channel pipette the solutions in the first row are mixed, then 100 µl per well removed and pipetted to the second row. This is continued up to row H and results in a 1:2 dilution for each row. The micro titer plates are incubated for 30 min at 60°C and measured in the ELIZA-reader at 560 nm. From the reference data (column 2) the protein concentration of the unknown probes can be derived immediately.

Electromobility Shift Assay (EMSA)

For electromobility shift assay the following oligonucleotides comprising NRF binding site were used:

NRE forward 5' AAT TCG AAT TCC TCT GAC GAA CA 3'
NRE revers 5' TGT TCG TCA GAG GAA TTC GAA TT 3'

As a control, double-stranded NF-κB consensus oligonucleotide (Santa Cruz) was used:

5' AGT TGA GGG GAC TTT CCC AGG C 3'
3' TCA ACT CCC CTG AAA GGG TCC G 5'

The oligonucleotides were end-labelled with ^{32}P-γATP by T4 polynucleotide kinase in the following reaction:

1 µl oligonucleotide (10pmol)
1 µl 10x polynucleotide kinase buffer
1 µl T4 polynucleotide kinase
2 µl ^{32}P-γATP (20uCi)
5 µl water

The labelling reaction was carried out for 30min at 37°C. And then unincorporated nucleotides were removed with a Sephadex G50 columns.
For annealing two complemented oligonucleotides were boiled for 5 min and then let slowly cool down to room temperature.
DNA binding:
Nuclear extracts (10 µg) were incubated for 30 min in binding buffer together with 1 x 10^5 cpm of labeled oligonucleotide.
Binding buffer: 50 mM KCl, 20 mM Tris-HCl pH 7,8, 2 mM Mg acetate and 1 mM DTT.
Samples were electrophoresed for 3 h on 5% polyacrylamide gels. Gels were dried and exposed to x-ray film for minimum 24 hours.

Western Blot

10-30 µg of protein per lane was separated on gradient 4-12% Bis-Tris gel (Invitrogen) and transferred by electroblotting onto nitrocellulose membranes (Amersham). The membranes were blocked in 5% non-fat dry milk dissolved in T-TBS (Tween tris buffered saline) (20 mM Tris/HCl, pH 7,5, 137 mM NaCl and 0,1% Tween-20). Immunoblotting was performed using monoclonal antibody directed against the Myc-tag (Roche) or polyclonal antibody directed against the 20 C-terminal amino acids of the NRF protein. Filters were then incubated with

the secondary horseradish peroxidase conjugated anti-mouse or anti-rabbit antibody. Proteins were detected using an enhanced chemiluminescence protein detection method (Pierce).

Immunofluorescence

Cells were plated onto 24 mm diameter round coverslips and fixed with cold (-20°C) methanol/acetone (1:1) for 5 min. After fixation, cells were washed 3 times with PBS containing 3% bovine serum albumin (BSA). The cells were incubated for 1 h at room temperature with the primary antibody (anti-Myc-tag or anti-NRF) at 1:250 dilution. Excess antibody was removed by washing 3 times with PBS containing 1% saponin. The cells were then incubated for 45 min at room temperature with fluorescent-labeled anti-mouse or anti-rabbit IgG antibody (Dianova) at 1:800 dilution. After washes with PBS 0,1% saponin cells were mounted onto glass slides with Elvanol and analysed.

Fluorescence Flow Cytometric Analysis

Fluorescence flow cytometric analysis was performed by a FACStar Plus apparatus (Becton-Dickinson & Co). Cells were detached by trypsin treatment. A cell suspension (500 µL) in PBS supplemented with 2% FCS was incubated with mAbs to be tested for 30 minutes at 4°C. Cells were then washed twice in PBS containing 2% FCS. The second incubation was carried out at 4°C with the use of FITC-conjugated goat anti-rat IgG (50 µg/mL, Jackson Immuno Research Laboratories, Inc). After 30 minutes, the cells were washed 3 times in PBS with 2% FCS and then analyzed.

Fluorescence Activated Cell Sorting (FACS)

This method was used to assess GFP expression by transfected cells. The cells to be tested were harvested (typically from well of 6-wellsplate plate), washed 1X with FACS buffer (1X PBS 2% FCS), spun at 1000 rpm for 5 min, resuspended in 500ul FACS buffer containing 10ug/ml propidium iodide to exclude dead cells and analyzed

Quantifying FACS Data:
FACS data collected by the computer can be displayed. The X-axis plots the intensity of green fluorescence. The individual black dots represent individual cells.

5.1 Plasmids and oligonucleotides

Existing plasmids that were used

muNRFtar -	M.Schwarzer, PhD thesis, 2001
pNRFrepaired -	I.Niedick, PhD thesis, 2004
pNRF(fl)-GFP -	I.Niedick, PhD thesis, 2004
pBS500 -	S.Gagneten at el, 1997
pM5ENRFN –	A. Otto, RDIF, GBF
N-TKmT -	Williams, 1988; Koblizek, 1997
(PymT expressing retrovirus)	kindly provided by Dr. Deutsch (University of Münster)

Plasmids cloned during the current work

pM5GFPCreneo -	retroviral vector carrying a 5' MPSV-LTR controlling a GFP-CRE fusion protein, followed by a Polyo-IRES element controlling a *neo* gene and terminated by a 3' MPCV-LTR (figure 2.25).
pM5mycNRFgtn -	retroviral vector carrying a 5' MPSV-LTR driving expression of a myc-tagged NRF protein, followed by a Polyo-IRES element controlling a *gfp-tk-neo* gene and terminated by a 3' MPCV-LTR (figure 2.33).
pM5mycNRFgfp -	identical to pM5mycNRFgtn but with exchange of *gfp-tk-neo* gene to eGFP gene.

Oligonucleotides

NRF 3'-end: 5' CTG AGA TAG GCT CCC GTA TGC CC 3'
NRF 5'-itr: 5' GTC TCT GGT ATA GCC TTA GTA GTG GG 3'
NRF 3'-out: 5' GTG ATG AGT GAC AGA GTC TGC TCC C 3'
Neo: 5' CCG CTT CCT CGT GCT TTA CGG 3'
IFNB_sense: 5' CAT CAA CTA TAA GCA GCT CCA 3'
IFNB_antisense: 5' TTC AAG TGG AGA GCA GTT GAG 3'
GAPDH_sense: 5' ACC ACA GTC CAT GCC ATC AC 3'
GAPDH_antisense: 5' TCC ACC ACC CTG TTG CTG TA 3'

5.2 Abbreviations

α	alpha
aa	amino acid
AB	antibody
ATG	translation start codon
ATCC	american type culture collection
ATP	adenosintriphosphate
β	beta
bp	basepare
cDNA	complementary DNA
CFU	colony forming units
Ci	Curie
CMV	cytomegalovirus
Cre	cyclization recombination
Da	Dalton
DBD	DNA-binding domain
DME	Dulbecco's Modification of Eagle's (medium)
DNA	desoxyribonucleic acid
DnaseI	desoxyribonuclease
dsRNA	double-stranded RNA
DTT	dithiotriotol
EB	embryonic body
E. coli	Escherichia coli
env	envelope gene
ES (cells)	embryonal stem cells
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
γ	gamma
GFP	green fluorescent protein
G418	aminoglycoside-2'-deoxystreptine (Gentamycin-derivative)
h	hour
HMGI(Y)	high mobility group protein
HTLV-1	human T Cell Leukaemia Virus
IFN	interferon
IL	interleukin
iNOS	inducible nitric-oxide synthase
IRES	internal ribosomal enty site
IRF	interferon regulatory factor
ISGF	interferon stimulated gene factor

ISRE	interferon stimulated response element
JAK	Janus-kinase
kb	kilobase
kDa	kilodalton
KO	knock out
loxP	locus of X-over (target sequence for the Cre recombinase)
LPS	lipopolysaccharide
LTR	long terminal repeat
MEF	murine embryonal fybroblasts
min	minute
MLV	murine leukemia virus
MOI	multiplicity of infection
MPSV	myeloproliferative sarcoma virus
mRNA	messenger RNA
n.d.	not determined
NDV	Newcastle Disease Virus
neo	neomycin phosphotransferase
NF-κB	nuclear factor kappa B
NLS	nuclear localization signal
NK	natural killer (cells)
NRE	negative regulatory element
NRF	NF-κB-repressing factor
NO	nitric oxide
NRD	negative regulatory domain
OD	optical density
PAGE	polyacrylamidgelelectrophoresis
PBS	phosphate buffer salt solution
PCR	polymerase chain reaction
PKR	dsRNA-dependent kinase
Polio	poliovirus
polyA	polyadenylation signal
PRD	positive regulatory domain
PymT	polyoma-virus middle T antigen
RNA	ribonucleic acid
Rnase	ribonuclease
rpm	rotations per minute
RT	reverse transcription
SA	splice acceptor
SD	splice donor
SDS	sodiumdodecylsulfate
Stat	signal transducer and activator of transcription
SV40	Simian virus 40

TGF	transforming growth factor
TLR	Toll-like receptor
TK	tymidine kinase
TNF	tumor necrosis factor
U	units
VEGF	vascular endothelial growth factor
VSV	vesicular stomatitis virus
WT	wild type

5.3 References

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